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SOME EFFECTS OF TEMPERATURE ON THE FREQUENCY OF DIVISION AND ON THE VOLUME OF STARCH AND FAT IN CHILOMONAS PARAMECIUM

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INTRODUCTION

The present experiments were undertaken to further the knowledge of the metabolism of *Chilomonas*, using temperature as the variable factor, and noting its effects on the frequency of division and on the volume of starch and fat.

Chilomonas paramecium is a small biflagellated protozoan of elliptical shape, filled with numerous round particles. It has been known since the publication of Ehrenberg (1838), in which it was described, pictured and named. Its particles were noted but incorrectly described as food vacuoles. Since then many investigators have contributed to the knowledge of this organism. Schneider (1854) recognized the starchy nature of some of the particles. Fisch (1885) and Dangeard (1910) described chilomonads and added briefly to the knowledge of the organism.

Mast and Pace (1932*a*, 1932*b*, 1933, 1935, 1937, 1938) made two important contributions to the study of *Chilomonas*. First, they developed methods for cultivating the species in a bacteria-free solution of inorganic salts plus sodium acetate; second, they discovered that some of the particles in the chilomonads were composed of fat. In their experiments they observed the frequency of division and the volume of starch and of fat in various solutions at a constant temperature.

Chilomonas possesses four properties that make it admirably suited for physiological experiments. First, using the methods of Mast and Pace, it can be grown in sterile solutions containing relatively simple solutes. Second, it divides at a fairly uniform rate. Third, it lives and

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thrives in a temperature range extending over twenty degrees. Fourth, it possesses a fairly uniform number of particles of starch and fat which are undoubtedly forms of stored food material.

Materials and Methods

The organisms were obtained from a strain grown in this laboratory for many years and cultivated exclusively in bacteria-free solutions containing sodium acetate and inorganic salts. In recent years, the strain had been kept at temperatures between 24° and 27° C.

Table I

Culture solutions for *Chilomonas paramecium*. The acetate ammonium solution is that of Mast and Pace (1938); the acetate-free solution is a modification free of nutritive substances.

	Acetate ammonium solution	Acetate-free solution
Sodium acetate, $NaC_2H_3O_2.3H_2O$	248.8 mg.	—
Ammonium chloride, NH_4Cl	46.0	46.0 mg.
Dipotassium hydrogen phosphate, K_2HPO_4	20.0	20.0
Ammonium sulphate, $(NH_4)_2SO_4$	10.0	10.0
Magnesium chloride, $MgCl_2$	1.0	1.0
Calcium chloride, $CaCl_2$	1.16	1.16
Sodium chloride, $NaCl$	—	112.5
Sodium sulphate, $Na_2SO_4 \cdot 7H_2O$	—	47.0
Distilled water	100.0 ml.	100.0 ml.

The culture solutions used in the experiments are described in Table I. The acetate ammonium solution is that of Mast and Pace (1938) and was used in all experiments requiring a nutrient medium; the acetate-free solution was used in experiments in which no food was supplied to the chilomonads.

The water used in making all solutions was redistilled in a Pyrex still and condenser and stored in Pyrex flasks until used.

The chemicals were Kahlbaum's " für Analyses " or " highest purity " except the sodium chloride which was Merck's " for biological purposes." The calcium and magnesium chlorides were weighed out approximately and dissolved in water, the molarity of the solution ascertained by the Mohr (1856) method for determination of chlorides, and the solutions

were then diluted to the required concentration. The required amounts of the other salts were weighed out and dissolved in the required volume of water.

Sterility of all solutions was maintained by heating in a hot air oven at 85° C. for 30 minutes on each of three days or by heating in an autoclave at 15 pounds pressure for 15 minutes. Cultures contaminated with bacteria or mold appeared occasionally but were discarded immediately and replaced by uncontaminated cultures. The results presented in this paper were obtained with chilomonads growing in sterile solutions.

All new slides and glass tubing were thoroughly cleaned by boiling in a strong aqueous solution of soap, rinsing, immersing in sulphuric-chromic acid cleaning fluid, and rinsing thoroughly in tap and distilled water. The culture slides were made of Pyrex glass and each had two depressions; these were placed in clean square Petri dishes, two slides to each dish, and the assembly sterilized in a hot air oven at 150° C. for 60 minutes.

Two types of pipettes were used, the "capillary pipettes" for isolating individual chilomonads, and the "measuring pipettes" for measuring and transferring solutions. Both types were made as follows: Pyrex tubing was cut into appropriate lengths, one end of each length was flanged to hold a medicine-dropper bulb and stoppered with cotton. The other end was drawn to a capillary point or calibrated to measure 0.1 cc. of fluid. Five such pipettes were put into test tubes, which were stoppered and sterilized at 150° C. for one hour. The pipettes were rinsed and sterilized after each usage.

Eight temperatures, each constant to $\pm 0.25°$ C., were available in a large rectangular bath. A compartment at one end contained a refrigerating unit, another at the other end a heating unit, and both were controlled by Aminco Metastatic thermoregulators. Stirrers provided a uniform temperature gradient between the two end compartments. Additional temperatures constant to $\pm 0.5°$ C. were available in a small bath.

In all experiments on the frequency of division the "isolation culture" method was employed. In this method, one chilomonad was isolated in a depression; the frequency of division was calculated from the number of progeny produced in a given time. Isolation cultures were prepared as follows. The hydrogen ion concentration of the acetate ammonium solution, prepared and sterilized as described above, was adjusted to pH 6.8 with sodium hydroxide or hydrochloric acid. A small quantity (0.1 cc.) was put into the depressions of clean, sterile slides in Petri dishes. A few cubic centimeters of water were poured into the bottom, and the dishes were put into the bath at constant temperature. After the temperature of the solution had reached that of the bath, one

chilomonad was introduced to each depression. The dish was left undisturbed for about 24 hours, and then the number of chilomonads in each depression ascertained. One of these chilomonads from each depression was transferred to the corresponding depression of a new dish and the process repeated as often as desired. The number of chilomonads in eight depressions was determined in each observation.

From the counts of chilomonads obtained in this manner, the frequency of division in divisions per hour was calculated as follows. The mean number of chilomonads per depression was computed. The number in the geometric progression of 2 immediately below the mean was subtracted from the mean; the remainder was divided by the same number in the geometric progression of 2 to give the fraction of divisions more than the last complete division. The result was added to the number of complete divisions. An example will elucidate the method. If 59 chilomonads were found in 8 depressions, then each had an average of 7.4 chilomonads. The number in the geometric progression of 2 immediately below 7.4 is 4, representing 2 complete divisions. Thus, 4 was subtracted from 7.4, and the difference, 3.4, divided by 4 to give 0.85 divisions more than the last complete division. The total number of divisions was 2 plus 0.85, or 2.85 divisions.[2] Convenient tables were constructed to abbreviate the calculations. The final value was last divided by the number of hours between observations to give divisions per hour. The method of computing the number of divisions is given here in detail because it differs radically from the method used by Mast and Pace (1933, 1935) for determining the frequency of division of chilomonads in isolation cultures.

There was much variation in the frequency of division and in the volume of starch and of fat under identical conditions of nutrition and temperature, so statistical methods were used to ascertain significant differences. The methods are those described in standard textbooks of statistics, such as Gavett (1925).

[2] It must be pointed out that the method described here is actually only a method of first approximation. The exact number of divisions can be determined from any number of organisms by applying the following equation:

$$X = \frac{\log N}{\log 2},$$

where N is the average number of chilomonads per depression, and X is the number of divisions.

Experimental Results

The Effect of Temperature on the Frequency of Division of Chilomonas paramecium

Chilomonads were grown exclusively in isolation cultures at all temperatures available, and the frequency of division measured. The results of this experiment are presented in Fig. 1. The figure shows that the frequency of division rises from zero at 9.5° C. to a maximum at 26°–30° C., and then decreases to zero at 35° C. Below 30° C. the measured frequency of division reflects correctly the rate of reproduction because the organisms live and thrive indefinitely. Above this temperature, however, the actual frequency of division may or may not

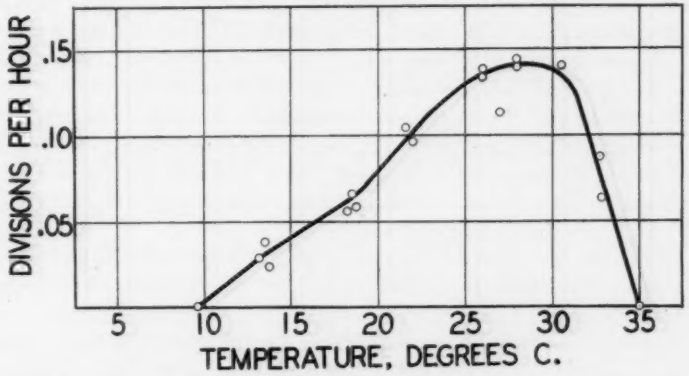

Fig. 1. The relation between temperature and the frequency of division of *Chilomonas paramecium*.

increase; the actual change is obscured by a decrease in the number of chilomonads brought about by death.

These experiments indicate that *Chilomonas*, like other living organisms, exhibits an increase in the metabolic activity (measured by the frequency of division) as the temperature rises to an optimum and then a decrease as the temperature rises still further. The reader is referred to Bělehrádek (1935) for an extensive review of this phenomenon.

An interesting observation was made in some preliminary experiments: for a short time after transfer of chilomonads from lower to higher temperatures, their frequency of division was low; gradually, however, it increased to that characteristic of the higher temperature. The experiment was repeated as follows to measure this phenomenon accurately and to seek other effects of low temperature on the frequency of division.

The organisms, apparatus, and materials were the same as in the preceding experiment. A clone derived from one chilomonad was grown at 27° C., and several from this clone were transferred to each of about 80 depressions and allowed to divide extensively at 27° C. These "stock cultures" were transferred to the compartment at 13.5° C. and left one week. Then isolation cultures were prepared and transferred to the six higher temperatures, where they were maintained and observed for two weeks. At the same time, the "stock cultures" were transferred to the compartment at 9.5° C. where they were kept for four weeks or until all chilomonads died. Isolation cultures were made from the "stock cultures" at intervals of one week, transferred to the seven higher temperatures, maintained and observed for a period of two weeks after establishment or, as was the case at 39° and 35° C., until all chilomonads died. The results are presented in detail in Figs. 2 and 3.

Figure 2 shows that during the first 48 hours after transfer from the low temperatures, the frequency of division increased from zero to that characteristic of the higher temperatures. Furthermore, after this period, the frequency of division remained constant at a value characteristic of the temperature studied, except that of those chilomonads exposed to 9.5° C. for 3 and 4 weeks, respectively, and observed at 30° C., which decreased to zero.

From this figure, three facts are evident. First, the exposure to low temperature modified the organisms so that their frequency of division was inhibited for a time after removal from the low temperature. Second, after the period of recovery from the effects of low temperature, the frequency of division became constant at a value characteristic of the temperature studied; in other words, exposure to low temperature had no permanent effect on the frequency of division. Third, all exposures to low temperature longer than one week had identical effects on the frequency of division at higher temperatures in which the chilomonads live indefinitely.

But what are the effects of exposure to low temperature on the frequency of division at temperatures in which the chilomonads do not live indefinitely? The following experiment answers this question.

Chilomonads were transferred from the "stock cultures" used in the preceding experiments to temperatures of 35° and 30° C. The chilomonads always die eventually at 35° independent of their treatment; at 30°, however, they normally live and flourish indefinitely. The results are presented in Fig. 3.

In the figure, the "percent surviving" was obtained by dividing the number of organisms living at the end of an observation period by the number introduced at the beginning and multiplying the result by 100.

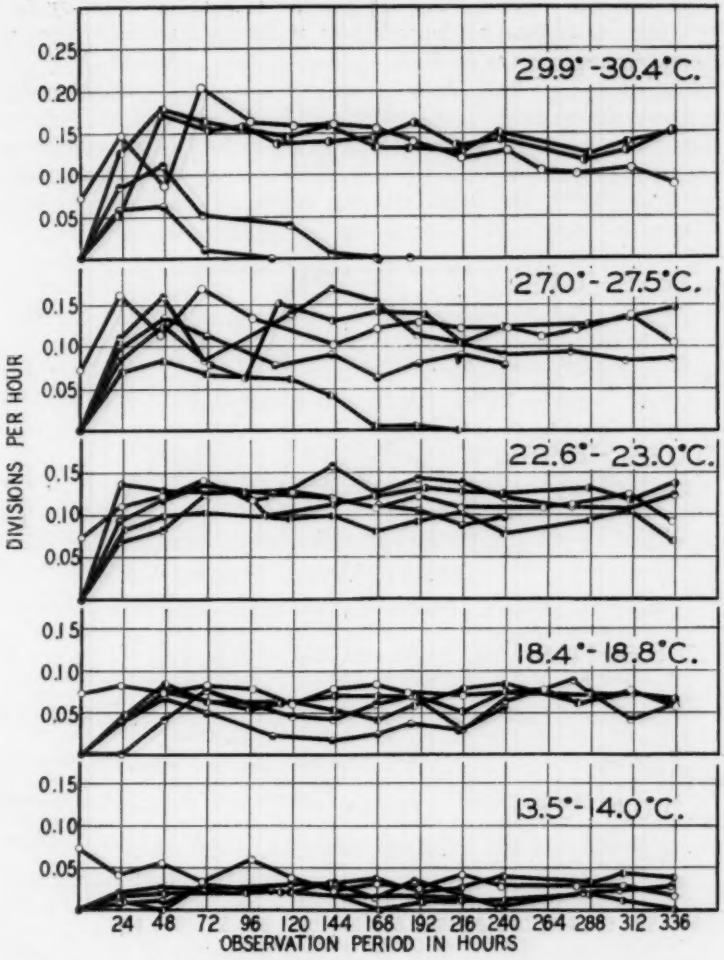

FIG. 2. The effects of prolonged exposures to low temperatures on the frequency of division at temperatures in which chilomonads live indefinitely.

○, exposed to 13.5° C. for 1 week.
◑, exposed to 13.5° C. for 1 week, then to 9.5° C. for 1 week.
◐, exposed to 13.5° C. for 1 week, then to 9.5° C. for 2 weeks.
◓, exposed to 13.5° C. for 1 week, then to 9.5° C. for 3 weeks.
◒, exposed to 13.5° C. for 1 week, then to 9.5° C. for 4 weeks.

Obviously, this "percent surviving" is the result of two opposing factors: the reproduction of the organisms on the one hand, and death of the organisms on the other hand.

Figure 3 shows that after the same period at 35° C., the percentage surviving decreased as the exposure to 9.5° C. increased. The figure also shows that after the various exposures to 9.5° C., the percentage surviving decreased as the exposure to 35° C. progressed. Furthermore, the figure shows that as the exposure to 9.5° C. increased, the time chilomonads lived at 35° C. decreased.

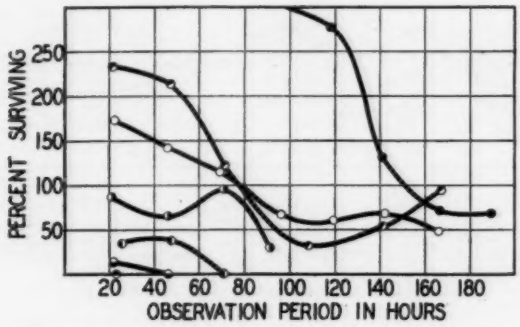

FIG. 3. The effects of prolonged exposures to low temperatures on the frequency of division at temperatures in which chilomonads do not live indefinitely.

○, exposed to 13.5° C. for 1 week, then observed at 35° C.

◑, exposed to 13.5° C. for 1 week, then to 9.5° C. for 1 week, then observed at 35° C.

◐, exposed to 13.5° C. for 1 week, then to 9.5° C. for 2 weeks, then observed at 35° C.

◓, exposed to 13.5° C. for 1 week, then to 9.5° C. for 3 weeks, then observed at 35° C.

◒, exposed to 13.5° C. for 1 week, then to 9.5° C. for 4 weeks, then observed at 35° C.

●, exposed to 13.5° C. for 1 week, then to 9.5° C. for 3 weeks, then observed at 30° C.

◴, exposed to 13.5° C. for 1 week, then to 9.5° C. for 4 weeks, then observed at 30° C.

It was noted above in Fig. 2 that the temperature of 30° C. acted as a viable temperature except when the chilomonads had been exposed to 9.5° C. for 3 and 4 weeks, respectively. Figure 3 also illustrates the effects of this low temperature on the percentage surviving at 30° C.: the same three phenomena evident at 35° C. appear. Experimental error accounts for the final rise in the percentage surviving of chilomonads subjected to 9.5° C. for 4 weeks.

Several conclusions may be drawn from these experiments. First,

exposure to low temperatures modifies the organisms in such a way that the frequency of division is low for a short time after transfer to higher temperatures. Second, the same modification apparently lessens the vitality of the organisms, because the ability to withstand detrimental effects of lethal high temperatures is reduced. Third, at temperatures in which the chilomonads live indefinitely the effects of the exposure to low temperature do not persist longer than 48 hours, but in other temperatures the organisms exhibit a decrease in survival that is in direct ratio with the length of exposure to low temperature.

Certainly the exposure to low temperature does modify the chilomonads. Modifications of this sort have been noted before. Dallinger (1887) induced modifications in certain monads that enabled them to live at very high temperatures and made it impossible for them to survive at the temperature in which unmodified monads flourished. Hance (1915) notes that a particular strain of *Paramecium caudatum* was able to resist higher temperatures than most and says that the former might have arisen in experiments dealing with high temperatures. Thus the present experiments serve to verify other observations, showing that organisms can be modified by environment and that the modification can persist for some time. The nature of the modification will be discussed again below under the effects of temperature on the changes in the volume of starch and fat.

The Effect of Temperature on the Volume of Starch and Fat in Chilomonas paramecium

The body of *Chilomonas paramecium* under conditions of optimum nutrition and temperature contains a fairly uniform number of particles; some of these give reactions characteristic of starch, some characteristic of fat. Under adverse conditions, the number and size of the particles may decrease or increase markedly. Mast and Pace (1932*a*, 1932*b*, 1933, 1935, 1937, 1938) make observations on the starch and fat of *Chilomonas* in solutions containing various substances always at one constant temperature. In their studies, they examined several chilomonads, then made a camera lucida drawing of a typical organism or of one taken at random. The present experiments were undertaken, first, to measure the actual volume of starch and fat in chilomonads, second, to observe the effects of different temperatures on the volume of starch and fat, and third, to ascertain the relation between the starch and fat and the frequency of division.

1. *In acetate ammonium solution.*—Experiments to ascertain the effects of various temperatures on the starch and fat under optimum conditions of nutrition were performed to supplement those of Mast

and Pace on starch and fat under various conditions at one constant temperature.

The organisms, solutions, chemicals, and glassware were precisely the same as in preceding experiments. The same methods were used to maintain sterility of the solutions, and the results appearing in this paper were obtained with solutions sterile except for the presence of the experimental chilomonads.

It was necessary to have a large number of organisms for each observation. To accomplish this several were transferred instead of the single chilomonad at establishment of each new culture.

The following procedure was used to measure the volume of starch and fat in *Chilomonas*. Several chilomonads from an experimental culture were drawn into a capillary pipette with the smallest volume of fluid; these were ejected on an ordinary glass slide, and surrounded by a ring of vaseline. Then one small drop of Lugol's solution and one of a saturated solution of Sudan III in absolute alcohol were added in succession with glass rods or wire loops. A cover-glass was placed over the drop and maneuvered to hold the drop in the center of the ring. This was observed with a microscope equipped with an ocular micrometer, and the size of each particle of starch and fat recorded. Five chilomonads were measured in each observation. Statistical methods were employed to ascertain significant differences.

Preliminary experiments showed that three temperature ranges could be distinguished: (*a*) the range between about 10°–13° C. and 30° C. in which the chilomonads live indefinitely (viable temperatures), (*b*) the range above the one in which the chilomonads live indefinitely (lethal high temperatures), and (*c*) the range below the one in which chilomonads live indefinitely (lethal low temperatures). Experiments were designed to show the effects of these three ranges on the volume of starch and fat.

a. Viable Temperatures.—The results of the experiments at viable temperatures are shown in the second and fourth columns of Table II. It is there shown that the volume of starch and fat per chilomonad at all viable temperatures is the same.

In order to obtain a more nearly complete analysis of the effects of viable temperature, the total volume of starch and fat synthesized by the progeny of one chilomonad in a 24-hour period was computed. This was done as follows. The number of divisions per 24 hours and thence the number of progeny produced from one chilomonad in that period were deduced from Fig. 1. By multiplying this number by the volume of starch and fat per chilomonad, the total volume of starch and fat synthesized in this period were obtained. The results are presented in

the third and fifth columns of Table II. It is there shown that as the temperature rises within the viable temperature range, the volume of starch and fat synthesized increases.

The remarkable part of these experiments is that although the rate of synthesis of starch and fat increases as the temperature rises within the viable temperature range, the volume of starch and fat *per individual* remains the same. One of two hypotheses may explain the situation. First, this may mean that under optimum conditions of salt concentration and nutrition in viable temperatures, the rate of synthesis of starch and fat controls the frequency of division. Second, the starch and fat may be by-products of metabolism, by-products which can be utilized as food materials under adverse conditions of nutrition, and their production and the frequency of division may take place at such rates that the volume of starch and fat remains constant.

TABLE II

The effect of viable temperatures on the volume of starch and fat per individual chilomonad and on the volume of starch and fat synthesized by the progeny of one chilomonad in a 24-hour period.

Temperature	Volume of starch per individual	Estimated volume of starch produced by progeny of one chilomonad in 24 hours	Volume of fat per individual	Estimated volume of fat produced by progeny of one chilomonad in 24 hours
30° C.	299.11±85.18	3806.87	52.07±20.12	701.67
27.3°	298.27±99.68	2907.53	66.92±22.48	652.08
23.2°	297.96±88.76	1348.15	65.99±32.76	298.43
18.5°	288.13±72.54	785.62	96.56±49.92	262.02
13.5°	314.18±61.15	518.86	119.50±34.01	197.34

b. Lethal High Temperatures.—The experiments were performed like those preceding, except that temperatures above the viable temperature range were used, that transfers were made at 24-hour intervals only when the experiment lasted several days, and that observations were made at 12-hour intervals. The results are presented in Fig. 4.

The figure shows that as the temperature rises above 32.5° C. the chilomonads lived for decreasing periods of time, and the volume of starch and fat increased as the exposure to the high temperature proceeded. This increase is due to two factors. First, the rate of synthesis either increased or was uninhibited. Second, there was a direct ratio between the length of life and the amount of accumulation. These two factors acted together to cause the greatest accumulation at 35° C.

It will be recalled that in experiments cited above, the frequency of division decreased as the temperature rose above the optimum; this, as

was pointed out, was largely due to the increase in the death rate and probably also to an actual decrease in the division. The present experiment demonstrates that the synthesis of starch and fat continues above 30° C., a temperature optimum for division, since starch and fat that would go into two or more dividing organisms now accumulate in one.

Thus the synthesis of starch and fat cannot control the frequency of division at lethal high temperatures. It is obvious that the frequency of division is inhibited whereas synthesis continues.

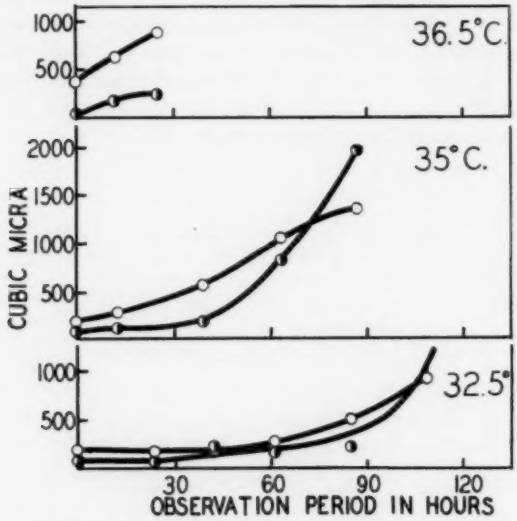

FIG. 4. The effect of three lethal high temperatures on the volume of starch and fat in *Chilomonas paramecium*.

○, volume of starch per chilomonad; ◑, volume of fat per chilomonad.

c. Lethal Low Temperatures.—These experiments were performed exactly as the preceding except as follows. A temperature of 9.5° C. was used since the organisms live for a considerable time but eventually die. Acetate ammonium solution was put into about 80 depressions, several chilomonads from a clone introduced to each, and the dishes put into the compartment at 27° C. for 24 hours until the chilomonads had become numerous. Then they were put into the compartment at 9.5° C. and observed periodically for 600 hours until the last chilomonad died. During this time the solution was replenished occasionally with fresh acetate ammonium solution. The results of this experiment are presented in Table III.

The table shows that as the period at 9.5° C. is prolonged, a decrease in the volume of starch is followed by an increase in the volume of fat. However, a statistical analysis shows that these changes are insignificant. The tendency for starch to decrease and fat to increase is, no doubt, present but is obscured by the great variation in the starch and fat in individuals under identical conditions.

It is quite probable that the modification of the frequency of division and the ability to survive at lethal high temperatures noted above as the result of prolonged exposure to low temperature is due to changes in the volume of starch and fat. However, experiments to be cited below indicate that the rate of resynthesis of starch and fat is so fast that the

TABLE III

The effect of a lethal low temperature, 9.6° C., on the volume of starch and fat per chilomonad.

Time at 9.6° C.	Volume of starch, cubic micra	Volume of fat, cubic micra
30 hours	165.4±35.9	35.9± 9.4
53	220.0±53.0	23.4± 14.0
90	138.8± 7.8	28.1± 3.1
124	37.4±25.0	57.7± 27.3
161	65.5±35.9	48.4± 39.0
211	28.1±25.0	129.5± 93.6
235	68.6±29.6	42.1± 18.7
292	51.5±10.9	84.2± 42.1
329	59.3±59.1	73.3± 25.0
353	106.1±26.5	73.3± 25.0
405	76.4±49.9	76.4± 31.2
497	71.8±54.6	173.2±138.8
521	99.8±64.0	248.0±126.4
598	64.0±28.3	224.6±163.8

modification can be accounted for only in part by changes in the starch and fat induced by low temperatures.

Since there is no division at this temperature and no increase in the volumes of starch and fat in the first 400 hours, there is no synthesis of these substances. Death could well be due to starvation in that no available food products are synthesized.

The decrease in the volume of starch followed by an increase in fat indicates that starch is probably transformed into fat.

2. *In Acetate-free Solution.*—Mast and Pace (1932*a*, 1932*b*, 1933) showed that if sodium acetate is omitted from solution, the volumes of starch and fat decrease to zero, and the chilomonads die of starvation. Their experiments were performed at one constant temperature, and they

made no actual numerical measurements of the volumes of starch and of fat. The present experiments were designed to ascertain the effects of temperature on the process of starvation of chilomonads in acetate-free solution and the effects of temperature on the recovery from starvation in acetate ammonium solution.

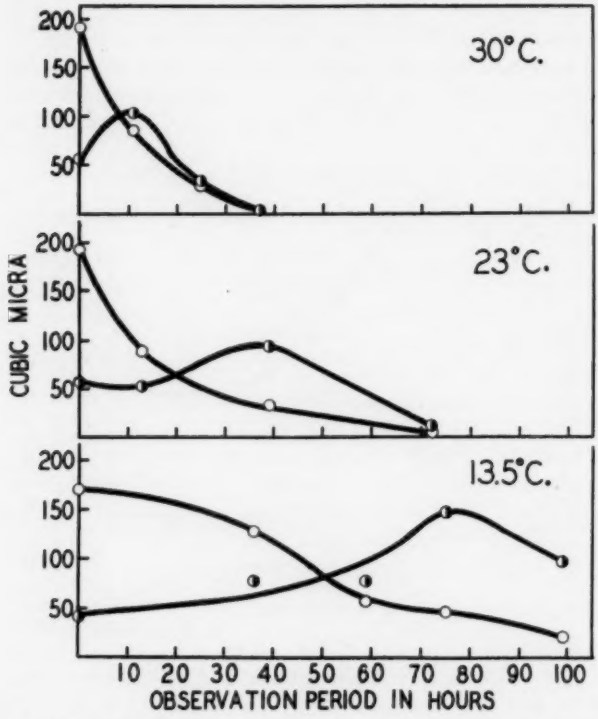

FIG. 5. The effect of viable temperatures on the change in the volume of starch and fat of chilomonads in acetate-free solution.

○, volume of starch per chilomonad; ◑, volume of fat per chilomonad.

Glassware and pipettes were the same as in the preceding experiments. The acetate-free solution described in Table I was used as the culture medium. The hydrogen ion concentration was adjusted to pH 6.8 with hydrochloric acid or potassium hydroxide, both prepared from boiled redistilled water. The Petri dishes were prepared as in the preceding experiments, except that a saturated solution of barium hydroxide

was poured into the bottom; this served to decrease the concentration of carbon dioxide in the atmosphere of the dish and to prevent the condensation of water beneath the depression slides.

A large number of chilomonads was transferred through four depressions containing acetate-free solution and then to the experimental acetate-free solution. It was then put into the constant temperature bath. No further transfers were made. The results of this experiment are presented in Fig. 5.

Figure 5 shows that the volume of starch decreased to a minimum, that the volume of fat first increased, then decreased to a minimum, and that these changes in the volumes of starch and of fat took place faster at higher than at lower temperatures.

Thus the same reaction occurs at the three temperatures studied; temperature acts as an agent that influenced the speed but not the processes involved in the reaction.

The changes noted here are similar to those indicated by the experiments cited above on the effects of lethal low temperatures. From these two sets of experiments, one can assume that under conditions in which there is no synthesis, the starch is gradually consumed, perhaps partially transformed into fat, and then the fat is consumed.

A second group of experiments was performed similar to the preceding, except that starved chilomonads obtained from cultures prepared as in the preceding experiment, were transferred directly to acetate ammonium solution. Synthesis of both starch and fat was rapid, requiring about 12 hours at 30°, about 15 hours at 23°, and about 18 hours at 13.5° C. for the volumes to increase from zero to the maximum typical of chilomonads grown in viable temperatures.

Discussion

The use of change in the volume of starch and fat as indicative of changes in metabolism of chilomonads raises three questions. First, is the method described in this paper sufficiently accurate to measure any changes in the volume of starch and fat? Second, is the change in the volume of starch and fat actually indicative of changed metabolism? Third, is the volume indicative of the mass of starch and fat?

The method is seldom very accurate. This is due to three factors: first, measurement of all particles is optically difficult; second, the particles are not always spherical, and their spherical diameter must be estimated; third, there is considerable variation in organisms kept under identical conditions. However, the method is undoubtedly more accurate than comparing organisms by means of camera lucida drawings or esti-

mating the relative amounts of starch and fat from a series of such drawings.

It is the author's contention that the volume of starch and fat may be indicative of the metabolic rate of the organism. The entire body of chilomonads living in acetate ammonium solutions, in natural ponds, or in rice cultures, is always well filled with starch and fat. This condition probably results from a balance between synthesis and decomposition of starch and fat; it is likely that in such an environment the chilomonads have a characteristic metabolic rate. A modification of the environment might upset this balance, causing a change in the rate of synthesis or decomposition, and subsequently, a change in the volume of starch and fat. Such changes would be accompanied by an alteration of the metabolic rate to a new and different characteristic value. Changes in the volume of starch and fat occurred and were described above; these were probably accompanied by changes in the metabolic rate.

The volume has been assumed in this paper to be an index of the mass of starch and fat. This is true if there are no changes in the chemical composition which alter the specific gravity of the starch and fat. Temperature is known to alter the iodine number of fats extracted from living organisms, but no experiments have been done on chilomonads to ascertain changes in the chemical composition of their protoplasm.

Summary

1. *Chilomonas paramecium* was grown in a solution of inorganic salts plus sodium acetate, and the effect of temperature on the frequency of division and on the volume of starch and fat measured.

2. As the temperature rises the frequency of division increases from zero at 9.5° C. to a maximum at 27°–30° C. and then decreases to zero.

3. When chilomonads are transferred from low temperatures to higher temperatures, a period of about 48 hours is required before the frequency of division increases to the value characteristic of the higher temperature.

4. When chilomonads are exposed to the low temperature of 9.5° C. for prolonged periods, then transferred to higher temperatures, the lethal high temperature and the period required to kill the organisms decrease. This indicates that the low temperature modifies the organisms and that the modified condition persists for some time.

5. At all viable temperatures, the volume of starch and fat per chilomonad remains constant, but as the temperature rises from 9.5° C., the volume of starch and fat synthesized increases, reaching a maximum at 30° C.

6. When organisms are exposed to lethal high temperatures there is an increase in the volume of both starch and fat. This is undoubtedly due to the fact that synthesis at these temperatures is not greatly impeded, that division decreases or ceases, but that the organisms live for a time and accumulate some starch and fat.

7. At lethal low temperatures, division ceases, there is a gradual decrease in the volume of starch accompanied by an insignificant increase in the volume of fat.

8. In solutions without sodium acetate, there is a decrease in the volume of starch to zero, an initial increase and a subsequent decrease in the volume of fat to zero. This process is identical at all viable temperatures but proceeds faster at the higher temperatures.

9. Synthesis of starch and fat by starved chilomonads in acetate ammonium solution is rapid and varies directly with the temperature.

10. The method of observing and measuring the volume of starch and fat has several drawbacks but does indicate changes with some accuracy.

11. The volume of starch and fat may indicate the metabolic rate of the organisms. Chilomonads living in an environment optimum for nutrition and temperature are filled with starch and fat and probably have a characteristic metabolic rate resulting from a balance in the rates of synthesis and decomposition of starch and fat. Changes in the environment may result in the breakdown of the balance, change in the volume of starch and fat, and probably a change in the metabolic rate.

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STUDIES ON EXPERIMENTAL HAPLOIDY IN SALAMANDER LARVAE

I. Experiments with Eggs of the Newt, Triturus pyrrhogaster

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Introduction

The problem of experimental haploidy has been investigated mainly in the Amphibia, since these eggs can quite easily be induced to begin development with the reduced number of chromosomes and the larvae are convenient material for microscopical studies.

In general, the results of many experiments using different methods and species have been that the development of the haploid embryos is abnormal. If development continues into larval stages, the animals usually, at one time or another, show certain symptoms of haploidy, namely, stunted growth, reduced activity, and an edematous swelling of the body. This is in direct contrast to the fact that natural haploids are known to exist in many species of arthropods and that viable experimental haploid plants have been produced. However, some haploid salamander larvae have been experimentally produced which were relatively free of some of the serious symptoms associated with haploidy (see review of the literature by Fankhauser, 1937). The most striking example was a metamorphosed *Triton* larva obtained by Baltzer (1922) and Fankhauser. Even though some of the activities of this larva were subnormal, so far as its internal morphology was concerned (Fankhauser, 1938*a*), the effects of the haploidy were not too serious.

It would be safe to conclude from the observations of Fankhauser on this single *Triton* larva that the morphological and physiological abnormalities which have been common to the majority of haploids need further investigation. The present experiments were undertaken, therefore, with the purpose of obtaining a number of advanced haploid larvae for a further study of haploid morphogenesis, and of examining the possibilities of experimental treatment of some of the serious physiological symptoms of haploidy in these animals.

The observations to be presented in this paper describe the animals which were obtained and the attempts to reduce the edematous swelling

of the body which appeared during the development of most of these larvae. Microscopical studies on the haploid animals will be published later.

The experiments were performed at Syracuse and many of the observations were carried out at the Marine Biological Laboratory, Woods Hole. A preliminary report of these experiments has already been published (Kaylor, 1939).

Material and Methods

Eggs of *Triturus pyrrhogaster* were selected for use, since, on the basis of Fankhauser's (1937) experiments on merogony with these eggs, it was believed that this species offered greater possibilities of extended haploid development than would others.

Eggs were obtained by anterior pituitary implantations. Development of the unoperated eggs was always normal. The female chromosomes were removed from the egg shortly after fertilization by means of a glass needle and small pipette (see puncturing method of Kaylor, 1937). All subsequent development then took place with only the male set of chromosomes. Control eggs, punctured to one side of the female nucleus, developed normally. These gave diploid embryos and larvae. Operated and control eggs were placed in a small dish of sterile modified Ringer solution,[1] and kept at a temperature of 20° to 22° C. until the animals had reached advanced larval stages (disappearance of yolk from the gut). From then on, the larvae were reared at room temperature varying between 22° and 26° C. Larvae were fed on *Enchytraeus*.

The corrosive sublimate, picric, acetic mixture of Michaelis was used for fixing yolk-laden embryos and larvae. Feeding larvae were fixed in Bouin's. In the sectioned material, Harris' hematoxylin was used for a nuclear stain and eosin or orange G in clove oil as a counter stain.

Observations

General Results (Table I)

Sixty-six out of 76, i.e., 87 per cent, of the punctured eggs of *Triturus pyrrhogaster* developed. The majority of these eggs were allowed to complete their developmental possibilities. Approximately 50 per cent of the developing eggs made at least an attempt at gastrulation, while 27 per cent developed beyond the gastrula stage. In a similar series of experiments with *Triturus viridescens* (Kaylor, 1937), only 10 per cent of all segmenting eggs developed beyond the gastrula stage.

[1] Solution according to Holtfreter except that the $NaHCO_3$ is omitted and the NaCl is reduced by one-half.

This comparison is of importance in experiments of this type where the main interest is in obtaining advanced stages of androgenetic development. It is also of interest to note from these observations that species differences are apparently an important factor in the extent and normality of haploid development, since in these two experiments the same methods were employed and the eggs were approximately the same size.

TABLE I

Androgenetic development of eggs of Triturus pyrrhogaster

Stage Reached	
No cleavage	10
Early cleavage stages	2
Late blastula	32
Gastrula	14
Neural plate	1
Neural tube	4
Neural tube, eye vesicles	1
Gill buds, limb buds, heart beat	7
Larva (30 days), three finger buds	1
Larva (47 days), hind limb buds	1
Larva (60 days), hind limbs four toes	1*
Larva (117 days), partly metamorphosed	1†
Larva (120 days), beginning metamorphosis	1‡
Total	76

* Killed by accident, not preserved.
† Died but preserved.
‡ Preserved while still developing.

Later Stages of Development

Evidence of Androgenetic Development.—The main interest of the present experiments was the group of 12 larvae which developed to stages ranging from the limb bud to a larva 120 days of age.

Seven of these larvae were preserved when they became edematous and failed to develop beyond the limb bud stage. Of the remaining five, which were the oldest larvae obtained, two were preserved because certain of their abnormalities were not compatible with further development, one (the oldest) was fixed while still developing, one was killed by accident some time after the first direct test of its chromosome condition, and one died at metamorphosis.

The evidence for the androgenetic development of these larvae is summarized in Table II. The retardation of development during gastrulation and neurulation has been established in other experiments (Kaylor,

1937; Porter, 1939) as a reliable criterion for the successful removal of the egg chromosomes.

TABLE II *

Evidence for androgenetic development of advanced larvae.

Stage of Fixation	Gastrulation and Neurulation	Pigment Cells	Chromosome Numbers
Limb bud, heart beat.....	Delayed	Small	—
Finger buds (30 days).....	Delayed	Small	12±1
Hind limb buds (47 days)...	Delayed	Small	12±1
Hind limbs (60 days).....	Delayed	Large	33+2–3
Larva (117 days).....	Delayed	Large	29+; 33+2–3
Larva (120 days).....	Delayed	Small	12±1; 24±2

* The chromosome numbers are the highest and the lowest in 20 or more counts made from tail fin epidermis and from various organs of the body, except in the case of the two triploids. In the 60-day triploid, there were three counts in all and only from the tail tip; in the 117-day animal, eleven counts from various regions of the body.

Description of the Three Most Advanced Androgenetic Larvae.— 1. The first larva, preserved on the 47th day (Fig. 1*b*), exhibited the typical symptoms of permanent haploidy: stunted growth, blunt head and small eyes, small gills, small pigment cells, sluggish reactions. During earlier development it had been edematous, which will be referred to in the discussion of edema, but at the time of fixation it was entirely

Figures 1 and 2, photographs of two of the oldest androgenetic larvae obtained: a haploid (Fig. 1), a haploid-diploid (Fig. 2). Figs. 3 and 4, photographs of two stages in the development of the haploid-diploid larva. Figs. 5, 6, 7, photographs of three stages in the development of the third advanced androgenetic larva: the triploid which died on the 117th day.

FIG. 1, haploid larva 47 days of age (*b*), diploid control of same age (*a*). Tail tip of both had been clipped. 4 ×.

FIG. 2, haploid-diploid larva 120 days of age (*b*), diploid control of same age (*a*). Tail tip of both had been clipped. *ca* 2 ×.

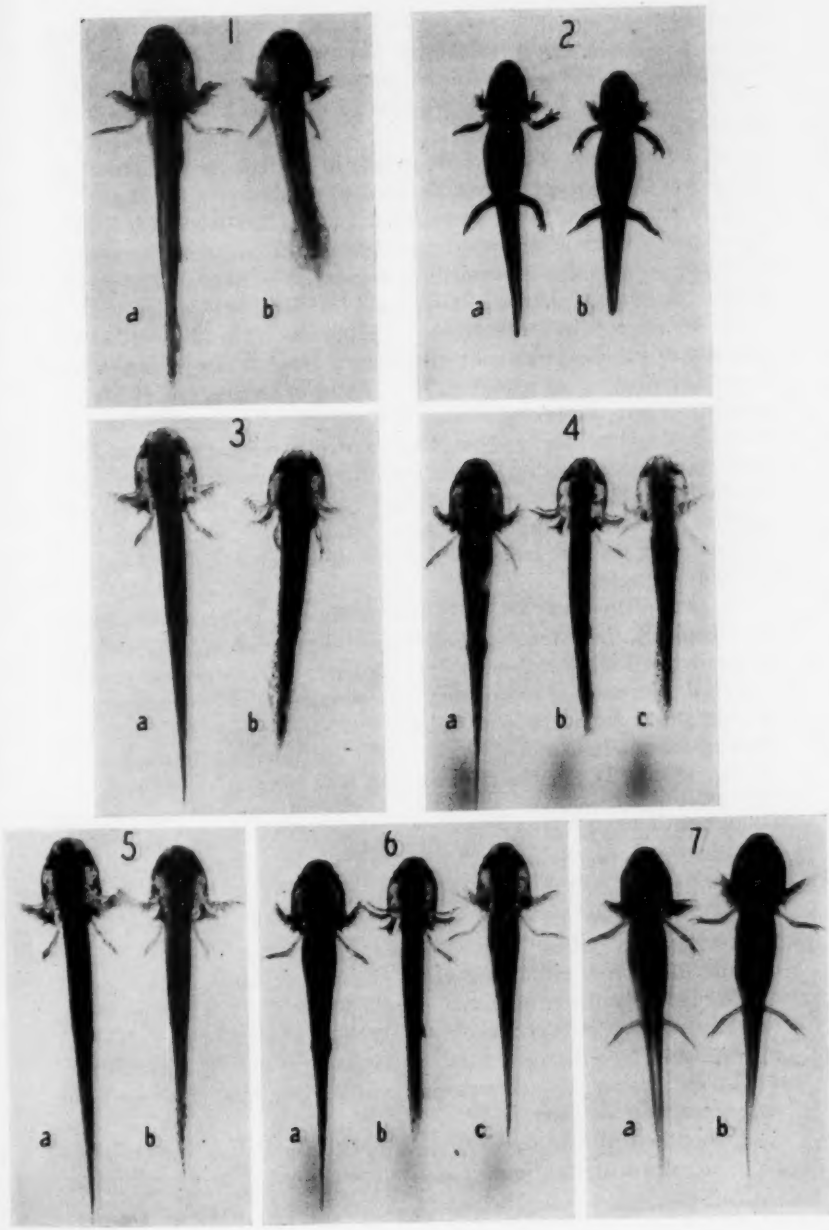
FIG. 3, the same haploid-diploid larva on the 21st day (*b*), diploid control of same age (*a*). Tail tip of *b* had been clipped. 4 ×.

FIG. 4, the same haploid-diploid larva on the 44th day (*c*), a diploid control about 3 days younger (*b*). Tail tip of both had been clipped. Diploid control (*a*) of same age as haploid-diploid larva. 2 ×.

FIG. 5, the triploid larva on the 21st day (*b*), a diploid control of same age (*a*). Tail tip of *b* had been clipped. 4 ×.

FIG. 6, the triploid larva on the 44th day (*c*), a diploid control about 3 days younger (*b*). Tail tip of both had been clipped. Diploid control (*a*) of same age as the triploid larva. 2 ×.

FIG. 7, the triploid larva on the 64th day (*b*), a diploid control of same age (*a*) with tail tip clipped. 2 ×.



FIGS. 1–7.

free of edematous swelling of the body which is so disastrous to most haploids. It was finally preserved, after it had exhausted the yolk supply in its gut, because its lower jaw was deformed and the animal was unable to take food. Introduction of macerated food through the mouth was unsuccessful.

All chromosome counts made so far in the tail fin epidermis and sections of the body have given the haploid number.

The existence of this larva demonstrates that advanced haploid larvae can be obtained with this method from whole eggs of this species. With larger numbers of animals it is not improbable that a greater number of advanced haploid larvae could be produced.

2. The second larva, fixed on the 120th day (Fig. 2*b*), unlike the majority of haploids, was not appreciably reduced in size in comparison to controls of the same age (Fig. 2*a*). Some of its morphological characteristics were, however, similar to those of other haploids, as, for example, the blunt nose and small head. In its reactions to stimuli the larva was as normal as any of the controls. This has not been a characteristic of all the other pure haploids obtained by a number of investigators. There had been an edematous condition of the belly and heart region during the limb bud stage, but this disappeared of its own accord and did not recur.

In spite of the fact that at the time of fixation this larva was unlike most haploids, there was the following evidence that it had developed with only the haploid male set of chromosomes: (1) A definite retardation in development at gastrulation and neurulation, which was evident in later stages (compare hind limb buds of this larva in Fig. 4*c* with a larva of the same age in Fig. 4*a* and with a control about 3 days younger in Fig. 4*b*). (2) Smaller pigment cells than those of controls (Figs. 3*b*, 4*c* compared to Figs. 3*a*, 4*a*, 4*b*). (3) The haploid chromosome number in ten nuclei of the tail tip epidermis of the young larva. Also the majority of epidermal nuclei (Fig. 8*a*) were smaller than diploid nuclei of controls (Fig. 8*b*).

However, when the entire animal was sectioned, preliminary chromosome counts in five different tissues showed that the larva was a mixture of haploid and diploid cells. Further investigation is needed to determine whether all organs are haploid-diploid. It seems probable, then, that regulation from haploidy to diploidy, which has been observed so frequently in parthenogenetically developed frog tadpoles (Parmenter, 1933, 1940; Porter, 1939; Kawamura, 1939), does occasionally occur in androgenesis in the newt.

Comparison of this larva (Fig. 2*b*) with the purely haploid larva (Fig. 1*b*) demonstrates, in part at least, the effect of haploidy-diploidy

upon the extent and normality of development. There is still the possibility, also, that a more normal morphological condition, which was independent of chromosome numbers, was obtained in this older larva.

3. A third larva died on the 117th day and was preserved. It had not completely metamorphosed. It was slightly larger than any of the controls of the same stage of development, possessed no apparent morphological irregularities, and was normally vigorous in all its reactions. No photograph was taken after the animal's death. However, in a photograph taken on the 64th day (Fig. 7*b*) it is seen that the larva appeared to be anatomically perfect and, even at this time, larger than controls of the same age (Fig. 7*a*). All larvae had been fed the same amount of food.

This larva was triploid, even though there was the suggestion that the animal had developed by means of androgenesis. The first prepara-

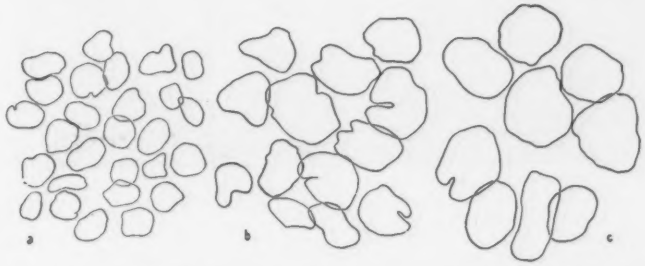


FIG. 8, camera lucida drawings of nuclei of epidermis cells of the haploid-diploid larva (*a*), of a diploid control (*b*), and of the triploid larva (*c*). 400 ×.

tion of tail fin epidermis offered 4 chromosome counts, all of which could be accepted as the triploid number. All nuclei in that piece of epidermis, a few of which are shown in Fig. 8*c*, were larger and less numerous than the diploid nuclei of controls (Fig. 8*b*). In a preliminary examination of sections of the entire larva, it was possible to make 7 chromosome counts from five different tissues (mesenchyme, cloacal epithelium, peritoneum, liver, heart). These counts were all approximately the triploid number, i.e., from 29 + to 33 + 2 — 3 (the triploid number being 36). It would appear, then, that this larva is entirely triploid and adds the eighth species to the list of Amphibia in which triploidy has been demonstrated.[2]

The evidence suggesting the origin of this triploid larva by means

[2] See review of literature by Fankhauser, 1938*b*; also see Kawamura, 1939, and Fankhauser, 1939.

of androgenesis is based on the following condition: a delay in development as compared to controls of the same age. The retardation of development, which was evident during gastrulation and neurulation, was still noticeable in the fore limb stage of the young triploid larva (Fig. 5*b* compared to Fig. 5*a*). Also the hind limb buds of this larva (Fig. 6*c*) were delayed in comparison to controls of the same age (Fig. 6*a*) and to controls about 3 days younger (Fig. 6*b*). Delayed development became less noticeable as the larva increased in age (Fig. 7*a* compared to Fig. 7*b*). The pigment cells were as large as or larger than those of the controls (as may be seen by magnifying Figs. 5*b*, 6*c* compared to Figs. 5*a*, 6*a*, 6*b*).

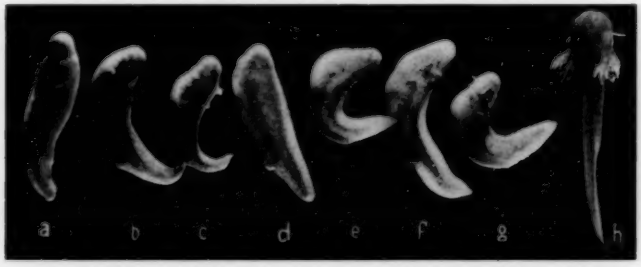


FIG. 9. Edematous haploid larvae (*a*, *b*, *c*, *d*, *e*, *f*, *g*) and a control (*h*) of the same age. Note the difference in size of the pigment cells of the haploid and diploid larvae. 4 ×.

There is no evidence as to the origin of triploidy in this case, but it must have occurred very early in development since only triploid chromosome numbers have so far been obtained in the entire larva.

Experiments to Reduce Edema.—The seven larvae which ceased development during the limb bud stage (Fig. 9, *a*, *b*, *c*, *d*, *e*, *f*, *g*) were edematous in the tissues of the heart and belly region. They were much smaller than controls (Fig. 9*h*) of the same age.

In two cases the edema began during the gill bud stage when pigment cells had appeared, the heart was beating, and Y-shaped blood islands were formed in the yolk region. In the other animals it appeared slightly later, at a time when circulation was established.

The development of the circulatory system of these larvae appeared to be abnormal, since in all cases there were localized accumulations of blood cells which persisted even after circulation had started in the rest of the body. This observation suggests that venous connection between the vitelline veins was not established, as Porter (1939) found in edematous frog larvae. The heart beat was normal. The pronephros

was probably established in all these larvae since the pronephric mound could be seen on the body surface. It is well established at this time in normal *Triturus pyrrhogaster* and venous connections of the cardinal veins are established in connection with the tubules. This suggests the functional activity of the tubules, since Armstrong (1932) has shown that in the fish, *Fundulus,* pronephric tubules become functional as soon as venous connections have developed in relation with the tubules.

As the edematous areas appeared they were opened under sterile conditions and the fluid allowed to escape. Then the animals were placed in sterile Clarke-Ringer solution.

In 6 cases, the edema returned within 12 to 24 hours in just as severe form as previously. The bloated areas were opened again and the larvae placed in sterile hypertonic salt solution but still the edema persisted. It gradually destroyed mesoderm and endoderm cells of the belly region and invaded the pericardial and head spaces. The heart action gradually became weaker until it was necessary to preserve the animals.

In the 7th larva (Fig. 9*a*), the edema returned in milder form and the animal lived for four days longer than the previous ones. However, its heart action gradually weakened and it was necessary to fix the animal. The head and fore limb region had differentiated slightly beyond the other six larvae.

The larva which was preserved on the 47th day (Fig. 1*a*) was also treated in the same manner for an edema which developed during the gill bud stage when circulation had been established. The condition disappeared completely and at the time of fixation it had not recurred.

A histological examination of all these larvae is in progress.

Discussion

The Production of Advanced Larvae

These results demonstrate that the majority of punctured eggs of *Triturus pyrrhogaster* made at least an attempt at development, and that a fairly high percentage of the segmenting eggs reached advanced stages of development.

It was also indicated that haploids of *Triturus pyrrhogaster* developed better than haploids of *Triturus viridescens.* Both species of eggs were practically the same size and were treated with identical methods. There are probably different factors in one species which determine the susceptibility of the developing eggs of that species to the effects of haploidy. Such variations between species (and genera) have been pointed out by other writers (P. Hertwig, 1923; Fankhauser, 1937; Porter, 1939).

However, the present experiments have produced no purely haploid

larvae which could equal the degree of differentiation obtained in the haploid *Triton* larva of Baltzer and Fankhauser. The reasons for this failure must await histological studies of the larvae which were obtained. There was the promising indication, on the other hand, that proper treatment of haploid larvae at critical periods in their development might possibly aid in lengthening the life of certain individuals.

One haploid-diploid larva was obtained which was apparently a normal individual since it developed to metamorphosis with no difficulty. It was pointed out that the success of extended development in this case may have been due to: (1) the normal number of chromosomes in many of its cells which may have affected physiological activities, and (2) a more normal morphological condition which occurred independent of the chromosome number.

Two triploid larvae were obtained in these experiments. All chromosome counts made so far in 5 different tissues of one larva have been triploid. The evidence for triploidy in the other larva is based solely on chromosome numbers and cell size in tail tip epidermis. The delay in development which was observed in both of these larvae might have been evidence of androgenesis. In this case, the triploid number of chromosomes must have originated in some manner from the sperm nucleus before first cleavage, because all chromosome numbers so far determined have been triploid. In order for this to have occurred it might be that the male haploid set of chromosomes divided once and then in some way one of these two sets divided again.

On the other hand, the delay in development might have been brought about by an entirely different set of circumstances: it may be that the female nucleus was left in the egg, retaining the second polar body (thereby giving a diploid female nucleus), and was then fertilized by the sperm nucleus. This would have produced a triploid embryo. It is impossible on the basis of the evidence at hand to say which of the two methods of origin of these triploid larvae was the most likely one.

Attempts to Reduce Edema

All haploids of these experiments suffered from edema at varying stages of development. In the majority of cases it was impossible to reduce this condition by the use of various concentrations of salt solutions. In only one larva was the edema permanently reduced by this treatment; in this case it is possible that the success was due primarily to a concomitant regulation of certain internal structures, such as the circulatory and kidney systems. Preliminary studies on the sections of this larva have indicated that this may indeed have been the case. Suc-

cessful treatment by these methods in any haploid larva, then, would depend upon the chance that certain internal structures are normally and even hyperplastically developed.

One other larva, the haploid-diploid, was edematous at an early period. The condition disappeared voluntarily. It is possible that further histological study of this larva may give a clue to the factors concerned in its improvement.

Aside from the observations on the living animals that the circulation was abnormal, no adequate explanations for the persistence of edema in these larvae can be advanced until the histological examination has been completed.

Summary

1. Eighty seven per cent of the punctured eggs of *Triturus pyrrhogaster* began development.

2. Advanced haploid larvae were obtained. Nine haploid larvae developed to stages ranging from the limb bud to a swimming larva 47 days of age. One haploid-diploid larva had begun to metamorphose at the time it was fixed.

3. Advanced androgenetic larvae can be obtained more readily in *Triturus pyrrhogaster* than in *Triturus viridescens: 27* per cent of the segmenting eggs developed beyond the gastrula stage in *Triturus pyrrhogaster* as compared to 10 per cent in *Triturus viridescens*.

4. Edema was the most common and serious abnormality associated with haploidy.

5. In the majority of cases it was impossible to reduce the edematous condition of the larvae with varying concentrations of salt solutions.

6. Two triploid larvae were obtained from punctured eggs. These larvae appeared to be entirely normal and slightly larger than the diploid larvae. This is the eighth species of amphibian in which triploidy has been observed.

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COMPARISON OF THE CHROMATOPHOROTROPIC ACTIVITY OF INSECT CORPORA CARDIACA WITH THAT OF CRUSTACEAN SINUS GLANDS[1]

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For a number of years it has been suspected from their histology that the corpora allata and the corpora cardiaca of insects have endocrine functions. Finally, in 1934, Wigglesworth demonstrated that tissues in the head of *Rhodnius* were responsible for humoral substances which were concerned with molt and metamorphosis, and later, in 1936, that growth and reproduction were also controlled to some extent by hormones. The corpora allata were suspected to be the source of the hormones concerned. Fraenkel (1935) demonstrated that a substance arising in the head of certain fly larvae was responsible for pupation in these forms. Pfeiffer (1939), by extirpation experiments, has shown that the gland concerned in the control of the activity of certain cells of the oviduct of the grasshopper was definitely the corpus allatum, as had been strongly suspected to be the gland concerned with the functions investigated both by Wigglesworth and by Fraenkel. Hanström (1936, 1937, and 1938) in a series of papers reported that the heads of insects, like the eyestalks of crustaceans, secreted a substance highly active in modifying the state of concentration and dispersion of crustacean chromatophores. In crustaceans Hanström had presented good support of the hypothesis that the sinus gland of the crustacean eyestalk was the source of the chromatophorotropic material. He was, however, unable to conclude just what was the source of the apparently comparable material from the insect head.

The greatest part of the work upon the insect endocrine glands has concerned the corpora allata. As yet no definite function has been ascribed to the corpora cardiaca. Recent work of Pfeiffer (1939) has given her some reason for suspecting that at least in *Melanoplus,* the corpora cardiaca or some structure located in the same region might be more directly concerned with molting in this form than are the corpora allata.

In the light of the work by Brown and Cunningham (1939) which

[1] This research was supported in part by a grant from the Graduate School of Northwestern University.

presented evidence that the sinus gland of *Cambarus* was directly concerned with control of molting in this form, and that of Brown (1940) showing that the sinus gland is also the eyestalk gland which produces chromatophorotropic hormones, it became interesting to discover whether in the insect as well, the same two functions (molt control and chromatophore activation) were to be found in a single gland. Were this true, then it would be the corpora allata to be first suspected since these were most generally believed to be the organs of the insect concerned with development.

Experimental

For these experiments the cockroach, *Periplaneta americana*, was used as the source of the insect tissues and the crayfish, *Cambarus immunis*, was the source of the crustacean tissues. After trying a number of methods of testing the action of various tissue extracts of the insect body upon crustacean chromatophores, it was finally decided to use isolated chromatophores as the test object. This practice was chosen inasmuch as it was possible to obtain great uniformity and sensitivity of response. The technic consisted of removing the carapace of a small crayfish (carapace length between 1.6 and 2.5 cm.) in the following manner: with fine scissors the carapace was cut from right to left just anterior to the cervical groove and then the scissors were inserted under the carapace in the median line at the posterior edge of the carapace in such a manner that the latter could be cut by a single stroke of the scissors into right and left halves. Each half was then removed by grasping the ventro-lateral edges of the carapace with forceps and lifting upwards, carefully tearing away the attaching muscles. Removing the carapace halves by grasping the dorsal cut edges invariably ruined the pieces. These two carapace halves, concave side uppermost, were placed in Syracuse watch-glasses, in sufficient Griffeth's solution to cover them. By rocking a sharp, round-pointed scalpel blade over them it was possible with a little practice to cut them up into a number of rectangular pieces (approximately 1×4 mm.) under a dissecting microscope. When viewed under a strong beam of light in an opaque white depression slide in a small quantity of Griffeth's solution with a compound microscope, it was possible to distinguish readily two types of chromatophores, red and white. These chromatophores were quite responsive to changes in the constitution of the bathing medium and retained this reactiveness for a number of hours. Furthermore, it was possible, by appropriate treatment of the crayfish which was to be the donor of the carapace, to obtain these two chromatophore types in almost any state of pigment dispersion or concentration. The sensitiveness of the isolated

chromatophores as test objects will become apparent in the experiments to be described.

Two difficulties arose in the use of these fragments of carapace in quantitative work. The first was that all of the chromatophores, either red or white, were not physiologically just alike. In the case of the darker red chromatophores there were those that were very sensitive to the dispersing and concentrating influences, and scattered in among these were smaller and paler red chromatophores which were somewhat less responsive. There appeared to be almost a complete series of grades of responsiveness. With the white chromatophores the situation was a little simpler since the few white chromatophores which were physiologically different from the majority lay in distinct patches. These patches were readily distinguishable since it was noted that these lay in the areas relatively free from red chromatophores and towards the dorsal portion of the carapace. By the proper selection of source of the test areas, these patches could be fairly well avoided. The second difficulty lay in the fact that at the greater dilutions of extracts tested, the chromatophores which were affected were more and more restricted to the peripheral region of the rectangular fragments, as if the material were diffusing inward from the cut edge.

In testing an extract sample, about 0.4 cc. of the sample was placed in a glazed porcelain depression and into this was placed one of the small carapace fragments. The general state of the red and white chromatophores was recorded at the beginning of the test and every fifteen minutes thereafter up to an hour. A control piece of carapace in Griffeth's solution was always kept for comparison. The state of the chromatophores was recorded in terms of a chromatophore index in which the chromatophore state at the time of any observation was expressed by a number ranging from one to five depending upon the degree of dispersion. A fully dispersed pigment mass was designated as 5 and a completely concentrated one as 1, with 2, 3, and 4 describing intermediate conditions. Due to the physiological differences of the chromatophores in a piece of integument, it was usually necessary to record a number describing the most fully dispersed chromatophores and another describing the most completely concentrated ones and then take the average value as the response to the extract tested.

The cockroaches used in the following experiments were all rather uniform in size ranging from 3.2 to 3.5 cm. in length (overall, excluding appendages). The crayfishes which served as donors of sinus glands varied in carapace length from 2.8 to 3.4 cm.

The Source of the Chromatophorotropic Hormones in the Insect Head

The first experiments were directed towards a localization of the source of the chromatophorotropic substance responsible for the results obtained by Hanström (1938). For this experiment the following tissues were carefully dissected out of the body of the cockroach: (1) brain; (2) frontal ganglion; (3) each of the three thoracic ganglia; (4) corpora cardiaca; (5) corpora allata. (Figure 1 shows a camera lucida outline of corpora allata and corpora cardiaca after dissection.) Each of the tissues was placed in a small porcelain depression. The brain was triturated and extracted in 0.8 cc. of Griffeth's solution; a single thoracic ganglion, the frontal ganglion, a single corpus allatum, and a single corpus cardiacum were each macerated and extracted in 0.4 cc. of Griffeth's solution. A piece of *Cambarus* carapace was added to

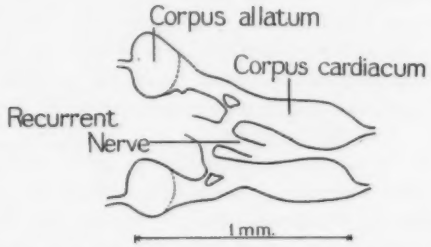

FIG. 1. A camera lucida outline of the isolated corpora allata and corpora cardiaca of the cockroach as seen from dorsal view.

each. Two Griffeth's solution controls were kept with each such experimental series. This procedure was repeated five times with the average results found in Fig. 2. The thoracic ganglia have been averaged together since there was not any perceptible difference found in the extracts of the three of them. It is noted that the only tissue of those tested which effected a concentration of the red pigment was the corpus cardiacum. On the other hand, it would appear from the data that brain and frontal ganglia were producing a concentration of the white pigment and that the thoracic ganglia were not doing likewise. Thus the concentrating agent of the brain and the frontal ganglion appears not to be a principle found in nervous tissue generally.

Brief experiments indicated that corpora cardiaca tissue extract was still strongly effective after boiling, and that the gland could be desiccated and stored for 24 hours without losing its capacity to concentrate red pigment when extracted.

The Size of the Cockroach Corpus Cardiacum

The cockroach corpus cardiacum was dissected out and outlined with the aid of a camera lucida. A rough approximation of its volume was made by constructing a clay replica of it and then reforming the clay into a sphere the volume of which was readily calculated from the diameter. Knowing the magnification of the model, it was easily found that

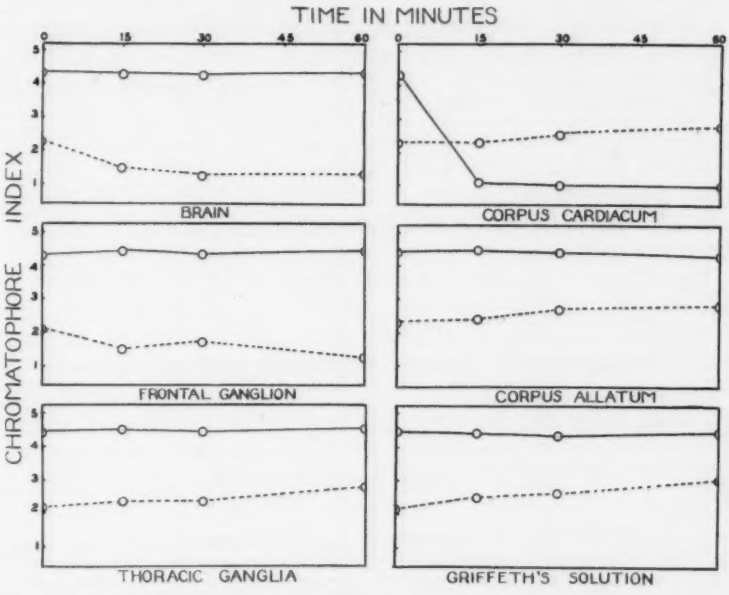

FIG. 2. The responses of red (solid line) and white (broken line) chromatophores of isolated pieces of crayfish integument to extracts of five organs of the cockroach body and to Griffeth's solution. A decreasing index with time indicates concentration of pigment and an increasing one, dispersion.

the volume of a single gland of the cockroach was about 0.019 mm.^{3}. The limits of the gland in making these measurements were determined by the extent of the bluish cast possessed by the gland when viewed in an intense beam of light. The glandular tissue appears more bluish than the neighboring nervous tissue and somewhat less transparent. In this respect, this gland resembles in appearance the sinus gland of the crayfish and other crustaceans.

On the basis of this rough determination it appeared that the corpus

cardiacum of our cockroaches was about half the volume of the crayfish sinus gland (Brown, 1940).

The Activities of Cockroach Corpora Cardiaca and Crayfish Sinus Glands as Seen in a Dilution Series

A long series of dilutions was made of each of the corpora cardiaca and the sinus glands and the effect of each member of the series was

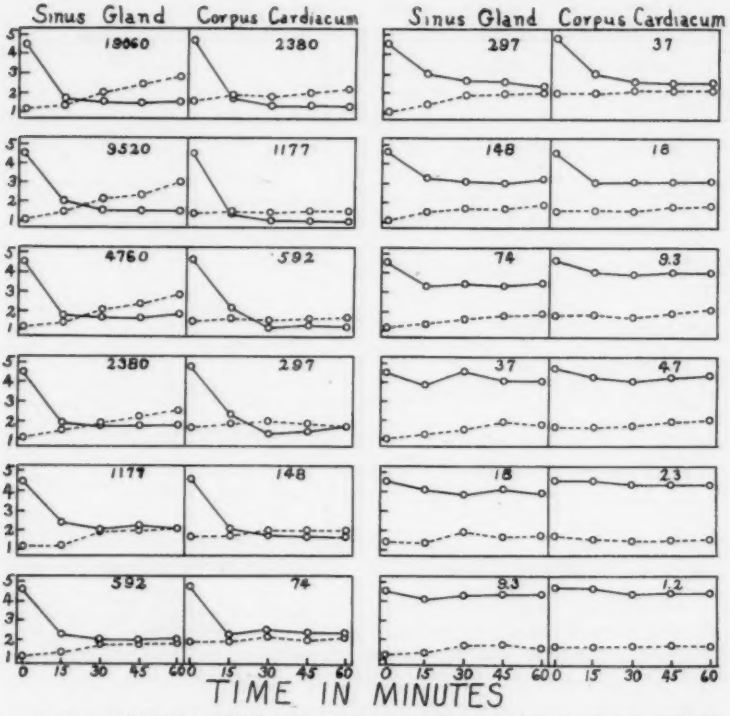

FIG. 3. The effect of dilution of the extracts of the insect corpora cardiaca and crustacean sinus glands upon red and white chromatophores. Red chromatophore responses are indicated by solid lines and white, by broken lines. The numerals along the ordinates are the average chromatophore indices. The numbers enclosed within the frames give the number of units of gland (by volume) to 2×10^4 units of solvent.

assayed in the usual manner upon isolated chromatophores. With such a method it was possible to discover the relative differences in the potency of the two tissues and at the same time to distinguish qualitative from quantitative differences in their activities.

For this experiment the corpora cardiaca were removed, permitted to dry briefly in a small mortar and then were triturated thoroughly in 0.8 cc. of Griffeth's solution. Half of this was used as the first sample to be tested and then the remainder was diluted to half the concentration and then half of this latter was set aside for testing. This procedure was repeated seventeen times, or in other words until the concentration was one part (by volume) of gland tissue to 2,760,000,000 parts of Griffeth's solution. It is important to point out that the dilutions were not made in the same vessel in which the glands were originally ground. Thus the results could not be influenced by a continued extraction of the original macerated tissue which might have adhered to the ground glass bottom of the mortar.

Exactly the same procedure was applied to the sinus glands of a crayfish. Two glands were originally extracted in 0.8 cc. of Griffeth's solution and diluted in steps with the concentration of each step being half that of the preceding one. Again here, seventeen such dilutions were made. Calculating on the basis that the volume of the sinus gland is about double that of the corpora cardiaca in animals of the sizes used, the maximum dilution obtained was one part gland to 1,380,000,000 parts of Griffeth's solution.

The above two experiments were repeated five times each. The greatest dilutions effective upon the red pigment for the experiments in which the corpora cardiaca were used were one part gland tissue by volume to:

11,050,000	parts	Griffeth's	solution
172,000,000	"	"	"
43,000,000	"	"	"
172,000,000	"	"	"
43,000,000	"	"	"

The average of these values is 88,200,000 parts of Griffeth's solution. In contrast to these, the last effective dilutions of the sinus glands fell at one part gland tissue to:

5,400,000	parts	Griffeth's	solution
2,700,000	"	"	"
43,000,000	"	"	"
1,347,000	"	"	"
21,600,000	"	"	"

The average of these values is 14,810,000 parts of Griffeth's solution. From this one would conclude that if the red pigment-concentrating substance is the same in the two glands the corpus cardiacum tissue con-

tains roughly six times as much active substance per unit volume as the sinus gland.

Figure 3 shows the comparative effects of dilutions of the corpora cardiaca and sinus glands upon the red and white chromatophores. A comparison of these two series yields some very interesting conclusions. (1) There is no significant effect of the extract of the corpora cardiaca upon the white chromatophores, while an extract of the sinus gland definitely dispersed the white pigment. (2) Volume for volume the corpora cardiaca tissue appears to be about eight times as effective upon the red chromatophores as the sinus gland.

Discussion

One interesting thing that these experiments indicate is that the crutacean sinus gland produces a concentration of the red pigment and simultaneously induces a dispersion of the white. At first this would appear to be a contradiction to the findings of Brown (1935) that eyestalk extract caused a concentration of the white when injected into eyestalkless *Palaemonetes*. The explanation of the difference is undoubtedly to be found in the great difference in the amount of eyestalk tissue involved in these two instances. Extracts of many crustacean central nervous organs is effective in concentrating white pigment.

Extracts of the central nervous system [2] of the crayfish are very effective in producing a concentration of both the red and the white pigment. In this we see a qualitative difference between the sinus gland and the nervous system since the former dispersed the white pigment. Although all parts of the crustacean nervous system are more or less effective upon both red and white pigment, the findings in this research indicate that the cockroach does not possess a red concentrating principle in any part of its brain or thoracic nerve cord or in its frontal ganglion, but does possess a white concentrating substance in its brain and frontal ganglion.[3] Thus it would appear that the chromatophorotropic substances which we have found in the nervous system are not materials of general distribution in the nervous tissue of all forms and hence are more likely to have a specific function of a humoral nature within the bodies of those forms in which they occur. The white chromatophores of most crustaceans respond to background changes and all the evidence at hand indicates that they are controlled in this reaction by hormones. Now, as far as we know, the only source of a concentrating principle for

[2] This effect is apparently not due to acetyl choline since experiments using a wide range of concentrations of this latter substance failed to give similar results.

[3] We have failed to find a red concentrating principle in the annelid nervous system.

this pigment is in the central nervous system, while an antagonistic (dispersing) substance is formed in the sinus gland. A comparable dual control of the black pigment in the telson and uropods of *Crago* is found (Brown and Ederstrom, 1940) in which the sinus gland produces a concentrating substance and part of the central nervous system (commissural ganglia) produces a dispersing substance. We are therefore led to suspect that here in the arthropods the nervous and endocrine mechanisms are more closely associated morphologically than in the higher vertebrates. Some of the hormones are produced by definite glandular bodies apart from the nervous system, while others are produced by cells intimately associated with nerve cells or perhaps possessing the dual function of secretion of hormones and conduction of nerve impulses.

The dilution experiments give us no reason for supposing that the red chromatophore-affecting materials from the insect corpora cardiaca and that from the crustacean sinus gland are not chemically the same. The material from the corpora cardiaca loses its effectiveness upon dilution at the same rate as that from the sinus gland. Furthermore, both withstand boiling and both retain their activity for some time in desiccated tissues. If these substances are the same, then a separate substance which disperses white must be present in the sinus gland.

Summary

1. The corpora cardiaca are the organs in the head of the insect which are responsible for the formation of a substance which produces a strong concentration of the pigment of the red chromatophores of crustaceans.

2. The corpora cardiaca still have an effect upon isolated red chromatophores of *Cambarus* when the gland is extracted in 80,000,000 times its volume of Griffeth's solution.

3. Neither the corpora cardiaca nor the corpora allata have any perceptible effect upon the white chromatophores of *Cambarus;* the latter gland is also without effect upon the red chromatophores.

4. The crustacean sinus glands still have an effect upon isolated red chromatophores when extracted in about 14,000,000 times their volume of Griffeth's solution.

5. The sinus glands of crayfishes produce a dispersion of the pigment of the white chromatophores of *Cambarus.*

6. The crustacean sinus gland and the insect corpora cardiaca differ qualitatively from one another with respect to their action upon the white chromatophores. With respect to the red pigment-affecting principle, the two glands are similar in that the rate of loss of effectiveness

with dilution is the same for both, boiling the extracts inactivates neither, and both glands retain their effectiveness for some time in a desiccated state.

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THE INDEPENDENT ORIGIN OF AMPHIBIAN RED CELLS AS SHOWN BY DIFFERENTIAL SUSCEPTIBILITY TO X-RAYS

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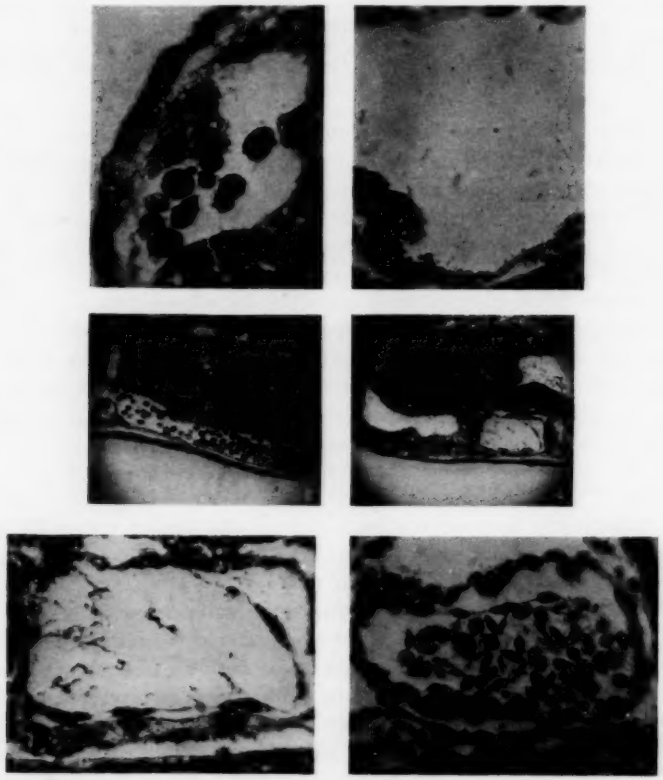
Knower (1907) described amphibian embryos in which normal blood cells and posterior circulatory channels developed after the heart anlage had been surgically removed. Interestingly enough, he noted in a single sentence the superficial similarity of the surviving operated embryos to certain X-rayed specimens. Federici (1926), Goss (1928), and Slonimski (1931) have reported successful surgical removal of the single large ventral blood island of early amphibian tadpoles and the subsequent development of normal, pulsating hearts and blood vessels without any circulating blood cells. The paper of Goss (*loc. cit.*) includes a translation of the account of the normal development of the blood island as given by Brachet (1921) and an excellent bibliography on the subject. Goss concluded that, since the excision of the blood island resulted as described above, "The angioblast concept cannot be applied to amphibians." In his experiments, performed with fine iridectomy scissors, it was necessary to remove a large part of the ventral region of the embryo with attendant loss of yolk, anomalies of the digestive system, and a very heavy operative mortality.

Stockard (1915) was able to obtain teleost embryos in which the normal erythrocytes developed in the blood islands and normal pulsating hearts were formed, although immersion in weak alcohol solutions prevented the connection of the hearts with the veins and forced the embryos to develop without circulation of the blood.

These experiments showed the separable, mutually exclusive character of the anlagen of the erythrocytes and heart at the very early stages used but did not define their places in the scale of differentiation. Among tadpoles of *Amblystoma jeffersonianum*, developed from embryos X-rayed at the early neurula (Harrison normal series, No. 14) stage, I have found embryos in which the specific dissimilarity of the

[1] Work carried on at the Marine Biological Laboratory under a Fellowship of the General Education Board. Now at the Department of Anatomy, University of Missouri.

embryonic differentiation of these anlagen is indicated. In such embryos the potential blood island cells are relatively so sensitive to X-ray injury that they may be completely inhibited by relatively low X-ray exposures (1000 *r*), while the anlagen of the heart and endothelium are uninjured and subsequently develop in a normal manner except that the colorless fluid which circulates is devoid of blood cells.



Photographs by Dr. Luther Smith, Research Associate, Field Crops Genetics, University of Missouri.

FIG. 1. Part of ventral blood sinus from normal 8-day embryo. × 75.

FIG. 2. Part of heart, same embryo as Fig. 1. × 75.

FIG. 3. Part of sagittal section of 12-day normal embryo. × 18.

FIG. 4. Part of sagittal section of 12-day embryo which had received 1000 *r* on the 4th day. × 18.

FIG. 5. Heart from Fig. 4, enlarged. × 75.

FIG. 6. Heart of normal 12-day embryo. × 75.

One thousand *r* were insufficient to inhibit the formation of heart and endothelium at the same stage, or any of the 5 other stages exposed and studied, so that the differences appear to be both quantitative and qualitative. Figure 1 is a photograph of a part of a sagittal section of a normal, 8-day embryo near the median plane. Part of the ventral blood sinus, formed at the site of the ventral blood island, is shown and the characteristic spherical, yolk-filled primitive blood cells are seen floating in their fluid or plasma. Figure 2 is an enlargement of the heart of the same animal with definite, continuous endothelial lining but no blood cells. Figure 3 is from a sagittal section of an embryo 4 days older showing the heart cavity and ventral blood sinus confluent and the primitive blood cells in all regions of the system. Note that at the caudal (right) end of the sinus part of the ventral blood island remains a compact mass. It can be readily observed that the ventral blood sinus is here supplied with an endothelium continuous with that of the heart (left). In Fig. 4 is shown a section of a 12-day embryo which had received 1,000 *r* 8 days earlier. The large heart (right) has a continuous endothelium but there is no endothelium lining the posterior cavity (left) in the blood island region and there are no blood cells. Figure 5 is an enlargement of the heart shown in Fig. 4 and Fig. 6 is a normal heart of the same age. It is crowded with red blood cells, many of which have changed from the primitive spherical type to the more advanced shape typical of the mature erythrocyte.

It appears from the study of these embryos and others from the same series that the anlage of the ventral blood island differs sufficiently from that of the heart and endothelium to allow X-ray destruction of the former without apparent harm to the latter. The bloodless condition was found in 9 of the 45 embryos studied. In 21 others the red cells were reduced in number and retarded in development.

In a separate series which received only 500 units of X-ray some specimens killed on the 20th day still show bloodless endothelial-lined hearts and unlined ventral blood sinuses not connected with the hearts.

It is concluded that the heart and endothelium of *Amblystoma jeffersonianum* arise from cells specifically distinct from the cells which produce the primitive blood cells. The difference is not a difference of degree of development or of position in the body but a true physiochemical difference. The red cells lost through inactivation of the blood island by X-rays are not replaced from any other source during the prehatching period.

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QUANTITATIVE STUDIES UPON SOME BLOOD CONSTITUENTS OF HELIX POMATIA

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(From the Laboratory of F. Holtz, Berlin-Frohnau and the Department of Biology, The Catholic University of America, Washington, D. C.)

The living activities of *Helix pomatia* are in many ways dependent upon the weather conditions. During the cold months, from about October or November to March or April, the snails hibernate, closing their shells by secretion of the calcareous epiphragma, and receiving their energy from stored reserve substances. During the warm months the intensity of their activity depends largely upon the amount of rain falling in any particular year, the snails feeding and being active only in moist surroundings with the period of reproduction lasting usually from June to August. In a previous investigation (v. Brand, 1931) it was shown that the chemical composition of the body of *Helix* shows characteristic differences at various times during the annual cycle. It seemed of interest to study some of the blood constituents, in order to test whether similar regular changes occur here.

Material and Methods

The animals, *Helix pomatia,* were purchased from a dealer in Franconia. They were kept in an enclosure in a garden and fed regularly with lettuce and cabbage leaves. Broken pieces of *Helix* shells were provided to ensure a sufficient calcium supply. The snails lived under these conditions indefinitely with few animals dying. Every year, however, a new lot of freshly collected snails was added.

Almost every month of the years 1933 to 1936 the blood of two lots of snails (10 to 12 animals each) was collected by puncture of the lateral vein. The following determinations were performed: dry substance by drying a sample at 100° C., total inorganic substances by incineration of dry substance, P according to Embden (1921), Ca according to Holtz (1934). The Ca analyses were usually performed on an incinerated blood sample, although preliminary tests showed that a direct precipitation of the calcium with oxalate, as practised in analyses on human blood, is also possible. In the latter case the snail blood has to be diluted with 4 to 5 volumes of distilled water, because of the large amounts of calcium present. Cl was determined according to Austin and van Slyke

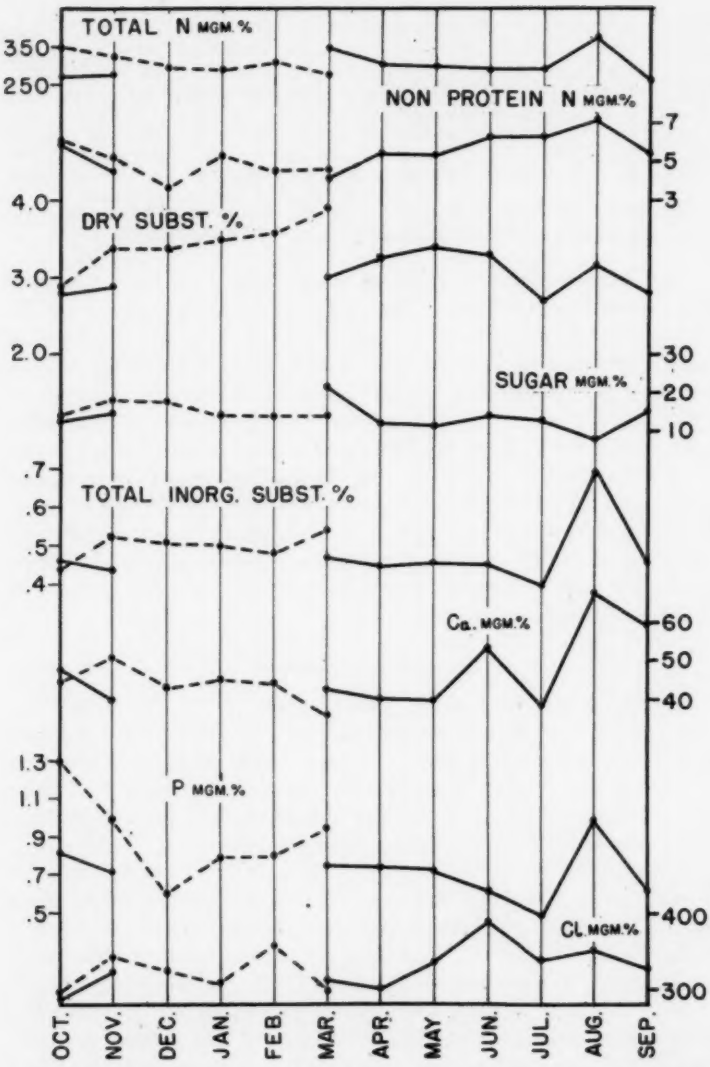

FIG. 1. Monthly averages of blood constituents of *Helix pomatia*. Solid line = Active life. Broken line = Hibernation.

(1920, 1921), sugar according to Hagedorn and Jensen (1923), total N and non-protein N according to a micro-Kjeldahl procedure. The values reproduced below for non-protein N are considerably lower than the figures of Wolf (1933), who reported values of roughly 20 mgm. per cent. This difference must be attributed to an incomplete precipitation of the proteins by Wolf. Dr. Wolf has since repeated his non-protein analyses in the laboratory of one of the authors (F. H.) and found the same low values that we give below. We used for the precipitation of the proteins a phosphomolybdenic reagent as prescribed by Bang (1927). Slightly higher values were found in preliminary experiments by using a mixture of tri-chlor-acetic acid with sodium-wolframate or, according to Folin, sodium-wolframate with ⅔ normal sulfuric acid.

TABLE I

Average values and maximal and minimal values of blood constituents of Helix pomatia.

	Average		Maximum		Minimum	
	Active life	Hibernation	Active life	Hibernation	Active life	Hibernation
Dry substance, *per cent*	3.1	3.4	3.9	4.0	2.0	2.4
Inorganic substances, *per cent*	.48	.50	.76	.63	.24	.40
Cl, *mgm. per cent*	331	322	450	395	220	265
P, *mgm. per cent*	.73	.91	1.80	1.50	.25	.36
Ca, *mgm. per cent*	48	44	133	82	28	7.9
Sugar, *mgm. per cent*	14	16	35	30	4	7
Total N, *mgm. per cent*	302	310	530	290	180	200
Non-protein N, *mgm. per cent*	5.6	4.9	14.6	8.4	4.4	3.0

Results

Fairly large variations of the monthly averages were apparent for all substances and they were in general decidedly more pronounced during the time of active life than during the period of hibernation (Fig. 1). Contrary to the findings reported previously for several constituents of the whole snail bodies, no significantly different trend of the blood constituent curves could be found during the different periods of the year. The extent of the variations present is demonstrated in Table I, where the maxima and minima both for the period of hibernation and of active life are shown. It is apparent that the minima are lower and the maxima are higher during active life. This is not surprising, since it is to be expected that with varying food and water intake during this time, the

composition of the blood will be more variable than during the period of hibernation.

It should be noted that many of the substances tested (i.e. total inorganic, Ca, P, total N and non-protein N) showed a peak for their monthly averages in August (Fig. 1). With the exception of non-protein N the highest absolute values (Table I) occurred in this month. The explanation lies in the fact that August is the hottest and dryest month of the year. A certain concentration of the blood may be expected to result from these climatic conditions. In agreement with this

TABLE II

Mean values of blood constituents of Helix pomatia during the formation of the epiphragma. Extreme values are given in parentheses.

	Active life	During process of secretion of epiphragma	After formation of epiphragma
Dry substance, *per cent*	2.35 (2.04–2.68)	3.08 (2.60–3.92)	3.41 (2.78–3.73)
Inorganic substances, *per cent*	.42 (.34–.56)	.50 (.42–.58)	.45 (.40–.48)
Cl, *mgm. per cent*	290 (184–391)	302 (270–354)	311 (263–348)
P, *mgm. per cent*	.73 (.56–1.0)	.94 (.67–1.1)	1.24 (1.1–1.4)
Ca, *mgm. per cent*	38.4 (35.5–43.0)	38.5 (32.5–49.5)	37.9 (34.5–44.0)
Sugar, *mgm. per cent*	15 (7–21)	17 (9–22)	16 (11–21)
Total N, *mgm. per cent*	272 (187–306)	327 (290–358)	358 (318–382)
Non-protein N, *mgm. per cent*	5.5 (4.2–8.1)	5.8 (4.6–7.3)	5.9 (3.3–8.4)

assumption is the fact that the blood sugar was found to be very low in August—in a dry month the chances are small that a snail will feed much. Curiously enough, Cl and total dry substance were neither especially high nor low during August.

It should be kept in mind that many irregularities of the curves will be due to accidental causes, for example whether the animals had or had not received rain during a few days, or even hours before the analyses. Such conditions can change the picture from year to year. It might be mentioned as an example that in one year the monthly maximum for total N was found in August; in another year the animals analyzed in the same month gave values similar to the lowest of this whole series.

In order to test whether or not a significant difference between the blood composition during the periods of hibernation and of active life exist, the monthly averages of both periods have been averaged. In this way the accidental causes due to weather conditions are thought to be eliminated for the most part. It is quite apparent (Table I) that the values for both periods are identical. It seems likely that these values, which are based on a large number of determinations, represent the normal average level of these substances in the blood of *Helix*. It seems necessary to assume that in *Helix*, as in higher animals, a regulation of blood constituents is present. But the levels are not as fixed as in the latter, as demonstrated by the variations mentioned above.

It has been stated previously (v. Brand, 1931) that about half the inorganic substance occurring in a snail in autumn is used for the formation of the epiphragma. At the same time much water is lost from the body. It seemed of interest to study the blood composition during this period and in the spring when the epiphragma is shed. The animals intended for these determinations were kept under close observation in the fall of 1935 and 1936. Three groups of individuals were analyzed: (1) animals leading an active life, (2) animals in the process of secreting the epiphragma, and (3) animals having just completed its formation. The three lots were analyzed on the same days. They had therefore been subjected to the same climatic conditions before the analyses. In the spring of 1936 and 1937 the blood composition of snails at the end of the hibernating period and after having shed the epiphragma was investigated in a similar way.

It is apparent that during formation of the epiphragma (Table II) the dry substance content of the blood rises. This is due largely to an increase of the protein concentration, as evidenced by the considerable increase in total N. Non-protein N and sugar were practically unchanged.

Both the total inorganic substance and two of its components, Cl and Ca, showed almost no change. P showed a more distinct increase. The fact that the inorganic substance showed no greater changes during the formation of the essentially inorganic epiphragma, is rather remarkable. The epiphragma is secreted by glands located at the edge of the mantle. An accumulation of inorganic substances there is known to occur long before the epiphragma is actually secreted. However, Barfurth (1883) showed that the inorganic substances of the liver are greatly reduced after the formation of the epiphragma. Obviously, it is to be expected that they were transported to the mantle edge by the blood. That no rise in the blood occurs, may be explained on the assumption

TABLE III

Mean values of blood constituents of Helix pomatia at the end of the hibernation period. Extreme values are given in parentheses.

	Before shedding the epiphragma	After shedding the epiphragma
Dry substance, *per cent*	3.87 (3.32–4.20)	2.85 (2.56–3.05)
Total inorganic substances, *per cent*	.53 (.48–.60)	.42 (.34–.51)
Cl, *mgm. per cent*	302 (270–319)	274 (248–286)
P, *mgm. per cent*	1.37 (.95–2.00)	1.07 (.45–2.00)
Ca, *mgm. per cent*	31 (23–37)	30 (18–40)
Sugar, *mgm. per cent*	13 (7–16)	35 (29–47)
Total N, *mgm. per cent*	386 (319–588)	327 (300–372)
Non-protein N, *mgm. per cent*	6.3 (4.8–9.2)	4.7 (3.4–6.5)

that these substances are deposited in the glands at the same rate as that at which they are mobilized in other tissues.

As soon as the epiphragma was removed in the spring and the snail resumed an active life, the concentration of most substances tested

TABLE IV

Effect of moisture and food on blood constituents of Helix pomatia.

	Lot 1 (moist surroundings + food)	Lot 2 (moist surroundings, no food)	Lot 3 (dry surroundings, no food)
Dry substance, *per cent*	2.98	3.02	3.06
Inorganic substances, *per cent*	.60	.63	.67
Total N, *mgm. per cent*	295	270	258
Non-protein N, *mgm. per cent*	1.9	1.5	1.3
Dry substance of snail body minus shell, *per cent*	22.6	21.5	22.8

dropped more or less (Table III). This is doubtless due to the fact that a snail sheds its epihragma in general only if the moisture in the surroundings is high, i.e. after a rain. It then has an opportunity to take in water at once. A corresponding observation concerning the total

body tissue has been published previously (v. Brand, 1931). It is curious to note, and as yet not explainable that the Ca content of the blood showed no change comparable to that of the other substances. The sugar content of the blood of free snails was found to have risen considerably. This may be interpreted as an alimentary hyperglycemia, since animals having shed the epiphragma begin immediately to feed.

Some experiments were performed in order to ascertain whether any blood constituents can be influenced readily under experimental conditions. They are of a rather preliminary nature, but are recorded here since we are at the present time not able to follow them up.

A short period of dryness, such as frequently occurs in nature, seems not to change the constitution of the blood to a marked degree. Fifty snails were divided into three lots as follows. Lot 1 was kept on moist

TABLE V

Blood sugar of Helix pomatia fed with cabbage soaked in 2 per cent dextrose solution.

Time	Blood Sugar *mgm. per cent*	Condition of Animals
0	8	Before feeding
1 hour	25	During "
2 hours	30	" "
3½ hours	49	" "
5½ hours	22	2 hrs. after feeding
7 hours	19	3½ " " "
9 hours	17	5½ " " "

filter paper with access to cabbage, lot 2 on moist filter paper but starving. Lot 3 was kept without food in an atmosphere dried by calcium chloride. The snails of lots 1 and 2 were active, those of lot 3 remained inside their shell most of the time, some closing the opening with a thin membrane. The blood composition after 5 days under these conditions is summarized in Table IV. There is no difference between the three lots.

The sugar content of the blood, on the other hand, can be influenced easily. A lot of snails was kept for some days without food or water. Then they were offered cabbage soaked in 2 per cent dextrose solution. They were allowed to feed for 3½ hours, following which they were again starved. At certain intervals during and after the feeding period the blood of some of the snails was collected and analyzed for sugar. A pronounced alimentary hyperglycemia was observed during the feeding period (Table V). The blood sugar sank slowly again as the snails

TABLE VI

Calcium content in normal and regenerating pieces of shell and in blood of Helix pomatia.

Lot	Days after removal of original piece	Ca Content		
		Removed piece *per cent total weight*	Regenerated piece *per cent total weight*	Blood *mgm. per cent*
1	0	—	—	38
2	7	44	10	94
3	14	34	33	42
4	24	36	32	34
5	34	35	?	32

were again starved, but it was still higher at the end of the experiment than at the beginning.

The last experiment deals with the question of whether the blood calcium rises when the snail is forced to regenerate a part of its shell. A fairly large piece of the shell was removed from some snails, and its Ca content was analyzed. After certain periods the Ca content of the piece regenerated and that of the blood was determined (Table VI). The Ca content in the regenerated piece reached the normal level after about two weeks. The value in lot 2 measured 7 days after the operation seems to indicate that the calcium content of the blood rises considerably in the early regeneration period. This is a contrast to the findings reported above concerning the formation of the equally calcareous epiphragma. Such a difference is not surprising. The formation of the epiphragma is a physiological process, whereas the regeneration of a large piece of shell is hardly a process to which the snail body is especially adapted. Small repairs, of course, occur frequently. Snails, for example, which had sealed together broken pieces of their shell, were fairly frequent in our material. We doubt, however, that under natural conditions snails would survive large defects to their shell.

Summary

1. Monthly analyses on different blood constituents of *Helix pomatia* showed that their concentration may vary considerably.

2. The mean values, gained from a large number of analyses, indicate, however, that there is no significant difference between the blood composition during the time of hibernation and that of active life. This is due to the fact that most, if not all, of the variability is due to climatic variations which balance out if observations are made over a long period

3. In snails forming the epiphragma the blood proteins and P were found in a certain concentration, but not the other inorganic substances.

4. The blood constituents become diluted by intake of water in snails shedding the epiphragma.

5. Short periods of dryness do not materially change the blood composition.

6. An alimentary hyperglycemia can be produced easily by feeding sugar to the snails.

7. In the early stages of shell regeneration the blood calcium is higher than normal.

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THE DISTRIBUTION OF INTERMEDIN: FIRST APPEARANCE OF THE HORMONE IN THE EARLY ONTOGENY OF RANA PIPIENS[1]

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Introduction

Extensive experimental work on the cytological picture of the pituitary gland, and statistical studies of the shifts in cell population of the adult organ have contributed materially to our knowledge of the physiology of the hypophysis. In the lower vertebrates, detailed studies of the histogenesis of the pars buccalis, which would be of significance morphologically and physiologically in relation to the sequence of events in ontogeny, have been relatively few; in the mammals, such investigations have been more extensive, although frequently made in a more fragmentary manner. Admittedly, integration of our knowledge of histogenesis with development of function in the pituitary gland is desirable, not only as an end in itself, but for explaining the phylogenetic significance of specific hormone effects. The chromatophorotropic hormone, intermedin, secreted by the pars intermedia, is interesting when considered in this respect. This hormone plays an important rôle in the regulation of physiological color changes in the lower vertebrates; in mammals, on the other hand, although large amounts of intermedin are present in the hypophysis, no definite function can be ascribed to this hormone, despite several attempts to investigate its part in the mammalian system.

In an earlier report (Kleinholz and Rahn, 1940) a method of assay for intermedin was developed, using the hypophysectomized lizard, *Anolis carolinensis,* as a biological test object. An attempt in that study to correlate the production of the chromatophorotropic principle in the pars anterior of the hypophysis of the chicken with a specific cell type did not yield thoroughly conclusive results because of the complexity of the material. The opinion was ventured that a physiological study of the ontogeny of intermedin might be more revealing, especially if studied in association with the appearance of cell types in the embryonic glands.

[1] These studies on the distribution of intermedin were aided by a research grant from the Permanent Science Fund of the American Academy of Arts and Sciences.

This report is an account of the ontogeny of the chromatophorotropic hormone of the pituitary gland in the frog, *Rana pipiens.*

Materials and Methods

Eggs of the frog, *Rana pipiens,* were obtained throughout the period of investigation by injecting mature females with extracts of triturated pituitary glands. The eggs thus secured were fertilized and allowed to develop at a room temperature of approximately 20° C. At various periods in the development, samples consisting of 250 to 300 individuals were removed for assay.

As is commonly known, even when precautions are taken to insure uniform environmental conditions, the eggs of a mass show considerable variation in the times at which they reach given embryological stages (Pollister and Moore, 1937). Table I shows the stages of the larvae at

Table I

Stages in the early ontogeny of R. pipiens taken for assay.

Stage	Age after fertilization	Stage number of Pollister and Moore
Four-cell	4 hours	4
Neural fold	52 hours	14
Tail-bud	96 hours	17
7-mm. larva	144 hours	20

the time they were taken for assay, and their age from the time of fertilization. In addition, unfertilized eggs from the ovary of a normal (uninjected) female killed in November were taken for testing.

The material to be used for assay was rinsed rapidly in two changes of pure acetone to remove excess water, after which the eggs and embryos were given a final change of 50 cc. of pure acetone for 24 hours. The unhatched stages (ovarian eggs, 4-cell and neural-fold batches) were dissected from their jelly or connective tissue capsules under acetone. The heads of the larvae in the tail-bud and 7-mm. stages, after the preliminary drying in acetone, were ablated (Fig. 1) and kept separately from the decapitated bodies which were to be used in the preparation of control extracts. The acetone-dried material was then dried in air at 40° C. for 3 days. At the end of this period the various embryonic stages were ground to a powder in a mortar and stored in a desiccator until used in the preparation of extracts.

The extracts were prepared by treating weighed samples of the dried material with N/10 NaOH, heating to the boiling point, neutralizing

with N/10 HCl against phenolphthalein as an indicator, and diluting to desired concentrations (from 3.6 to 9.0 mg. dry weight of powder per 1.0 cc.) with cold-blooded Ringer's solution. The stock extracts thus prepared were placed in ampules and capped, then immersed in boiling water for 15 minutes, after which they were stored at 1° C. No preservatives were added.

The prepared extracts were injected as 0.2 cc. samples intraperitoneally into each of 10 hypophysectomized lizards, and the degree of dispersion of the pigment within the dermal melanophores was measured on a numerical scale of five stages of the total color range (see Kleinholz and Rahn, 1940). For the bionomics and physiology of metachrosis in this animal, reference may be made to an earlier study (Kleinholz, 1938).

Observations

The data obtained from these experiments are arranged in Tables II and III. In Table II are shown the concentrations of the prepared extracts and the amounts of injected material from the embryonic and larval stages. Table III shows the melanophore responses of the groups of test animals to injection of the various extracts.

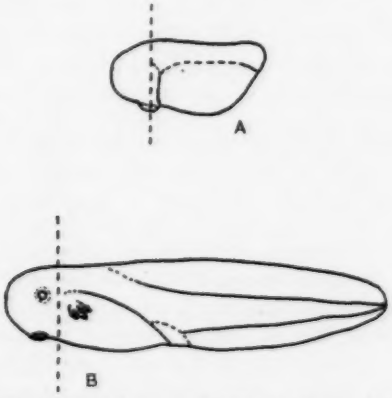

Fig. 1. (*A*) The tail-bud larva and (*B*) the 7 mm. free-swimming larva. The dashed vertical lines show the amount of cephalic material used for the preparation of the extracts.

It is evident from an examination of these tables that ovarian eggs, eggs in the four-cell stage and in the neural-fold stage contain no intermedin and therefore have no effect on the dermal melanophores of the test animals, even though injected as extracts of comparatively high con-

centration. The first positive response of the melanophores was obtained with extracts prepared from heads of larvae in the tail-bud stage. The average response for 30 injected animals was 1.3 ± 0.9 on the scale of the chromatic range. As defined in the previous study (Kleinholz and Rahn, 1940), the *Anolis* unit (A.U.) for intermedin is that weight of pituitary powder or its equivalent which will evoke an average stage 1 response in a group of 10 injected test animals. Since each of the injected animals received the equivalent of 4 heads of larvae in the tail-bud stage (Table II), each head therefore contains approximately 0.32 A.U. of intermedin. Control injection of extracts prepared from the de-

TABLE II

Concentration of the prepared extracts and the amount of tissue injected into each test animal.

Extract no.	Nature of extracted material	Number used	Dry weight	Conc. of extract (wt. dry powder in 1.0 cc.)	Amount of tissue injected into each animal
			mg.	*mg.*	
1	Ovarian eggs	250	252.9	7.2	1.4 eggs
2	Four-cell stage	210	207.2	7.2	1.4 eggs
3	Neural-fold stage	260	265.6	9.0	1.8 eggs
4*a*	Heads of tail-bud larvae	240	43.6	3.6	4.0 heads
4*b*	Bodies of tail-bud larvae	240	184.6	3.6	0.9 body
5*a*	Heads of 7-mm. free-swimming larvae	264	40.5	3.6	4.8 heads
5*b*	Bodies of 7-mm. free-swimming larvae	264	198.6	3.6	0.9 body
6	Heads of hypophysectomized 7-mm. free-swimming larvae	50	—	—	5.0 heads

capitated bodies of these larvae, in equal amounts and concentrations, gave no detectable response. Similarly, extracts prepared from the heads of 7-mm. free-swimming larvae upon injection yielded an average response of 1.7 ± 1.1 for 30 lizards, indicating an approximate intermedin content of 0.35 A.U. per head in this developmental stage. Considering the fact that these are biological tests made with comparatively crude materials, the values for the amount of intermedin in the heads of the two larval stages are in very good agreement and indicate the sensitivity of the test in the assay of minute quantities of biological material. Control injections made with extracts prepared from the decapitated bodies of these 7-mm. larvae were completely without effect.

These results where the first detectable amounts of intermedin in the

frog larva appear during the tail-bud stage of development are readily correlated with the appearance of the hypophyseal primordium as an ingrowth of the buccal ectoderm. To eliminate the remaining possibility that some cephalic structure outside of the hypophyseal primordium was responsible for the intermedin effect, frog larvae in the tail-bud stage were hypophysectomized according to the method of Smith (1916). These animals were allowed to grow to 7-mm. in length, at which time the heads of 50 larvae were separated from the bodies and prepared as

TABLE III

Melanophore responses of the test animals to injection of extracts.

Extract number	Dry weight of powder injected as extract into each animal	Number of animals injected	Response
	mg.		
1	1.44	9	0
2	1.44	10	0
3	1.80	10	0
3	1.80	10	0
4*a*	0.72	10	0.9±0.7
4*a*	0.72	10	1.2±1.0
4*a*	0.72	10	1.9±0.9
4*a* (average)	0.72	30	1.3±0.9
4*b*	0.72	10	0
5*a*	0.72	10	1.7±1.1
5*a*	0.72	10	1.6±1.2
5*a*	0.72	10	1.7±1.2
5*a* (average)	0.72	30	1.7±1.1
5*b*	0.72	10	0
6	0.72*	10	0

* Five heads were injected into each animal. From Table II, this amount is estimated to weigh approximately the figure given.

before. The extracts prepared from these hypophysectomized heads contained no detectable amounts of intermedin.

DISCUSSION

An attempted correlation between ontogeny of intermedin and the cytological differentiation of the pituitary gland in *R. pipiens* is seen to be pointless. The first detectable traces of intermedin in the embryology of the frog occur at the tail-bud stage, where the ectodermal hypophysis has only recently become invaginated to lie below the infundibulum. Although at this stage the cells of the pituitary demonstrate a well-initiated physiological differentiation, there is no cytological differentiation. His-

tological examination of the hypophyseal primordium from the tail-bud larva reveals embryonic ectodermal cells containing scanty, non-staining cytoplasm, frequently with pigment (melanin) granules which have presumably been derived from the egg pigment by passive enclosure during cleavage. No chromophillic granules are present and the pars intermedia is not differentiated. Kerr (1939) describes a very similar condition for the newly-hatched larva of *R. temporaria.* According to this author, the first eosinophiles appear in the anterior pituitary of larvae which are 11–13 mm. in length, while "in the intermediate (lobe) of the frog a few scattered basophiles are to be found" in the 32 mm. tadpole which has hind and fore legs and tail fully developed.

Several studies of the pituitary gland have been made integrating cytological development with the development of physiological activity. Outside of this report and the work of Kerr (1939) there are, however, only a few other studies on the differentiation of the pars intermedia and the appearance of detectable amounts of intermedin. Snyder (1928) detected qualitatively the presence of intermedin in pituitary glands from pig embryos of 30 mm. crown-rump length. Both Maurer and Lewis (1922) and Nelson (1933) found that the first secretory granules in the pars intermedia of the pig appeared in embryos 175 mm. in length. The former authors, however, connected this histological differentiation with the appearance of the pressor principle of the pituitary gland.

Intermedin is thus seen, from the results cited above, to be one of the earliest hormones formed by the pituitary gland. Certainly, in the frog, the first detectable amounts of this hormone appear with the formation of the pituitary primordium. The physiological significance of this early appearance of intermedin is probably to be correlated with the onset in larvae of *R. pipiens* of physiological color changes about 10 days after hatching. The absence of metachrosis in the early larval stages (4–7 mm. length) of this species is undoubtedly due to the fact that morphological differentiation has lagged behind physiological differentiation; that is, the integumentary melanophores, the eye, and the optic pathways to the brain and pars intermedia have not yet become fully established.

Summary

1. Quantitative assays for the first appearance of intermedin in larvae of *R. pipiens* were made, using the hypophysectomized lizard, *Anolis carolinensis,* as test animal.

2. Intermedin is detectable with the establishment of the hypophyseal primordium as an invagination from the buccal ectoderm. The gland from larvae 4–7 mm. in length contains approximately 0.3 *Anolis* unit of intermedin.

3. Intermedin appears before cytological differentiation of the pituitary gland occurs.

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STUDIES ON THE TREMATODE FAMILY MICROPHALLIDAE TRAVASSOS, 1921

IV. The Life Cycle and Ecology of Gynaecotyla nassicola (Cable and Hunninen, 1938) Yamaguti, 1939

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Introduction

In the first of this series of studies the writer (1939) described *Cornucopula sippiwissettensis* gen. et sp. nov. from shore birds near Woods Hole, Mass. Since then, an investigation into the life cycle has shown that *Cercaria nassicola* Cable and Hunninen, 1938, is the larval stage of this trematode. Yamaguti (1939) established a new genus, *Gynaecotyla,* to include species formerly placed in the genus *Levinseniella* but having characters incompatible with those described for *Levinseniella.* Since Yamaguti's paper appeared while the writer's was in press, his generic name has priority; likewise, Cable and Hunninen's specific name has priority; therefore, the correct name of this microphallid trematode is *Gynaecotyla nassicola* (Cable and Hunninen, 1938) Yamaguti, 1939.

Until the brief report by the writer (1939*a*) of the life cycle of *Cornucopula nassicola* (now *G. nassicola*), no complete life history of a microphallid trematode was known. Numerous reports have indicated larval stages in various genera of this group. Reference to the first three papers of this series (Rankin, 1939, 1939*b*, 1940) and to the papers of Hadley and Castle (1940) and Cable and Hunninen (1940) will eliminate unnecessary discussion here of the literature on this subject.

The present paper is an elaboration of the original brief report (1939*a*), presenting an experimental proof of the life cycle of *Gynaecotyla nassicola* with notes on the ecology of this parasite.

It is unfortunate that this genus must again be emended. But since several important characters are either interpreted wrongly or are mentioned as absent when actually they are present, a restatement of the generic diagnosis seems pertinent. In the first place, Yamaguti (1934, 1939) described a female genital sucker lying between the acetabulum and the cirrus or testis; this structure has been shown by the writer (1939) to be a second ventral sucker, with no connection whatsoever

with the genital system. Secondly, Yamaguti indicated that there is no receptaculum seminis or Laurer's canal; actually, these structures are present and can be found even in the metacercaria. Finally, several writers have included this genus in the family Heterophyidae. As will be discussed later, a study of the morphology and life history indicates that *Gynaecotyla* belongs to the family Microphallidae.

Gynaecotyla Yamaguti, 1939, char. emend.
Syn.: *Cornucopula* Rankin, 1939

General Diagnosis.—Microphallidae Travassos, 1921. Very small, pear-shaped trematodes with spiny cuticula; anterior end bluntly tapering, posterior end broadly rounded. Two ventral suckers located at beginning of posterior body third. Prepharynx, pharynx, and esophagus present; intestinal crura usually extend to posterior level of ventral suckers. Male copulatory organ dextral, consisting of a complicated muscular structure lying in the genital atrium; it is composed of two recurved horn-like projections, the tips of which usually bear spines; pars prostatica weakly developed. Uterus enters genital atrium at lateral edge of dextral acetabulum, close to the common genital opening. Large transverse cirrus pouch, containing bulbous seminal vesicle and coiled ductus ejaculatorius, lies anterior to the ventral suckers. Ovary sinistral, lateral to and on same level as ventral suckers. Testes symmetrical, just posterior to the ventral suckers. Uterine coils filling space behind testes; may extend in front of testes to anterior edge of suckers. Laurer's canal and receptaculum seminis present. Vitellaria follicular, in two compact groups, one behind each testis. Excretory bladder V-shaped; flame-cell pattern, 2 $[(2+2)+(2+2)]$. Adults in intestine and caeca of shore birds; metacercariae in crustaceans. Type species: *Gynaecotyla squatarolae* (Yamaguti, 1934) Yamaguti, 1939.

Syn.: *Levinseniella squatarolae* Yamaguti, 1934
Cornucopula squatarolae (Yamaguti, 1934) Rankin, 1939

Other species include:

1. *Gynaecotyla jägerskioldi* (Travassos, 1921)
   Syn.: *Levinseniella jägerskioldi* Travassos, 1921
   Cornucopula jägerskioldi (Travassos, 1921) Rankin, 1939
2. *Gynaecotyla simillimus* (Travassos, 1921)
   Syn.: *Levinseniella simillimus* Travassos, 1921
   Cornucopula simillimus (Travassos, 1921) Rankin, 1939
3. *Gynaecotyla adunca* (Linton, 1905)
   Syn.: *Distomum aduncum* Linton, 1905

Levinseniella adunca (Linton, 1905) Linton, 1928
Cornucopula adunca (Linton, 1905) Rankin, 1939

4. *Gynaecotyla nassicola* (Cable and Hunninen, 1938) Yamaguti, 1939
Syn.: *Cornucopula sippiwissettensis* Rankin, 1939
Cornucopula nassicola (Cable and Hunninen, 1938) Rankin, 1939

Figures and descriptions of all five species may be found in the writer's 1939 paper.

Materials and Methods

Living material was used as much as possible, because many structures, particularly gland ducts and flame cells, cannot be observed in preserved specimens. Neutral red was used successfully as an intravitam stain. Material for mounting whole was fixed in Conant's fixative (50 per cent Alc., 100 cc.; Formalin, 6.5 cc.; glacial acetic acid 2.5 cc.), stained in Grenacher's Borax-Carmine precipitated with HCl, and mounted in damar. Material for sectioning was fixed in Bouin's fixative, stained in Delafield's haematoxylin, and counterstained with eosin.

Snails from the infected locality were isolated in fingerbowls to obtain cercariae; crustaceans were examined for metacercariae. In order to obtain parasite-free hosts for experimental infection, snails and crustaceans from widely separated localities were isolated and examined. Young gulls, used as experimental definitive hosts, were taken from the nest when they were four or five days old and kept on a diet of fish and squid.

All drawings were made with the aid of the camera lucida, small details filled in by free hand. All measurements given below are in millimeters.

Field Observations

While studying the trematode parasites of shore birds in the Woods Hole region, it was observed that these birds were feeding largely, if not entirely, on crustaceans. Stomach contents invariably consisted of digested or partially digested specimens of *Talorchestia, Orchestia, Gammarus,* etc. The following crustaceans were collected and examined for metacercariae: *Talorchestia longicornis* (Say), *Orchestia platensis* Kröyer, *Chirodotea caeca* (Say), *Gammarus locusta* (Linn.), *Haustorius arenarius* (Slabber), *Crago septemspinosus* (Say), *Palaemonetes vulgaris* (Say), and *Virbius zostericola* S. L. Smith. In *Talorchestia longicornis* alone were trematode cysts found and these cysts contained metacercariae that appeared morphologically like the adult *Gynaecotyla*

nassicola found in the birds. These metacercariae were fed to young gulls and in a few days examination of these experimental hosts produced adult *G. nassicola,* thereby establishing the identity of the metacercariae.

The only snails occurring in abundance in this area are *Littorina littorea* (Linn.) and *Nassa obsoleta* (Say). Thousands of these were isolated. A tiny microphallid cercaria was found in *Nassa,* none in *Littorina.* This was identified as *Cercaria nassicola* described by Cable and Hunninen (1938, 1940). Experimental evidence has yielded positive proof of the identity of this cercaria as the larval stage of *Gynaecotyla nassicola.*

Experiments Proving the Life Cycle

Penetration of Cercaria into Crustacean Host

Several specimens of *Nassa obsoleta* shedding *Cercaria nassicola* were placed in fingerbowls with *Talorchestia longicornis,* since this was the only species of crustacean found naturally infected. Controls were kept at all times. Examinations were made at regular intervals of both experimental and control animals. Negative results were obtained. Most of the crustaceans were either killed by the snails or drowned. Since this cercaria is the only one of its kind in the locality, the negative results were puzzling. A study of the habits of *Talorchestia* indicated that this amphipod lives in the sand *near* the water line, not *in* the water. In fact, when a fingerbowl had sand in it rising above the water level, the talorchestiae always climbed out of the water on to the sand. This suggested another set of experiments. Three dishes, six inches in diameter and three inches deep, were used. Sand to the depth of one-half inch was placed in each dish with a small mound of it to one side. Water was added to cover the bottom sand, but the mound was left above the water level. Three specimens of *Nassa obsoleta* shedding cercariae were placed in each dish, along with ten *Talorchestia* from an uninfected locality. The water was changed morning and night by running sea water into the sand, thoroughly washing it. This simulated the diurnal changes occurring on the beach. One hundred per cent infection was obtained; the controls proved negative. The first crustacean was examined five days after exposure and contained nine tiny cysts. A corresponding increase in number and size of cysts was found with longer exposure. A maximum of 93 cysts, with an average of 22 per host, was obtained. In nature, an average of 6 cysts, with a maximum of 26, was found in 100 specimens of *Talorchestia* examined. The cysts are found free in the pericardial cavity, not embedded in tissues as is characteristic of other

members of this family (Cable and Hunninen, 1940; Hadley and Castle, 1940).

The method of penetration is quite different from that reported for *Spelotrema nicolli*. In the latter, the crab becomes infected in the water by sweeping the cercariae into the gill chamber where the larvae penetrate into the efferent veins. Since *Talorchestia* is not equipped with this type of respiratory mechanism, a different mode of entrance must be sought. Also, since these amphipods died when left in water alone, another method must be used. Cercariae without tails were found actively moving about in the branchial lamellae (Fig. 1) attached to the second gnathopods and first, second, third, and fourth periopods. Some were found boring through the tissues towards the dorsal pericardial cavity. These tiny cercariae, then, like so many of the microscopic sand-dwelling species, can live in the moist sand between the tides and can penetrate the amphipods as the latter lie quiescent below the surface. The sand- and mud-burrowing habit of the snail host also aids in completing this stage of the worm's life cycle.

Specimens of *Haustorius arenaria, Chirodotea caeca, Emerita talpoida,* and *Orchestia platensis* were exposed in a manner similar to that above. Not one of these animals became infected.

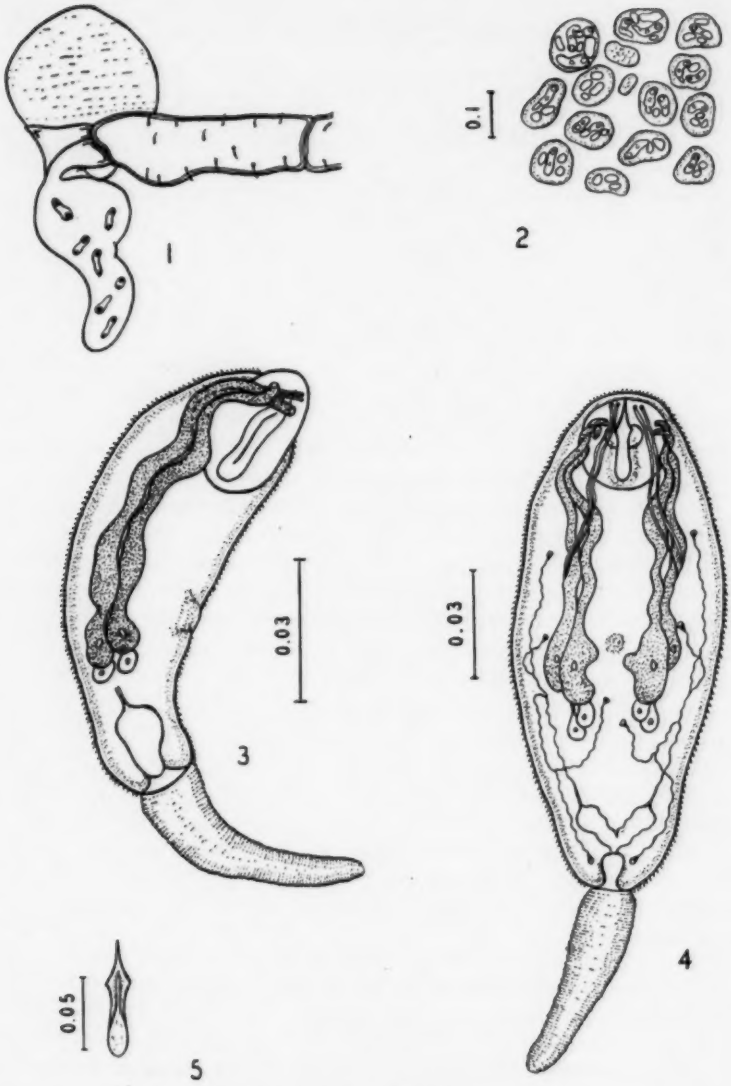
Infection of Definitive Host

Fifty mature cysts collected from naturally infected talorchestiae were fed to one young herring gull (*Larus argentatus*). Two days later the bird was killed and examined. Twenty-one immature adults of *Gynaecotyla nassicola* were obtained, some with eggs present in the uterus. Sixty-five cysts from experimentally infected talorchestiae were fed to a second gull, and 36 to a third. Thirty-five adults were obtained on examination of the former host seven days later, and 15 from the latter eight days later. Two control birds were negative for this species of trematode.

Description of Stages in the Life Cycle

Sporocyst (Fig. 2)

The cercariae develop in small oval, thin-walled sporocysts averaging 0.1 in diameter. Many specimens of *Nassa obsoleta* were found to be heavily infected, the livers completely riddled with these larvae. Very few cercariae or germ balls are present, a maximum of 8 cercariae, with an average of 6 being found. A study of sectioned material indicates that two or more sporocyst generations may occur.



EXPLANATION OF PLATE I

FIG. 1. Proximal end of second gnathopod of experimentally infected *Talorchestia longicornis*. Note tail-less cercaria that have penetrated the branchial lamella.

FIG. 2. Cross-section through sporocysts in the liver of *Nassa*.

FIG. 3. Lateral view of cercaria showing openings of glands, unusual thickness of gland ducts, position of stylet, and primordium of ventral suckers.

FIG. 4. Ventral view of cercaria showing arrangement of glands and excretory pattern.

FIG. 5. Stylet of cercaria.

Cercaria (*Figs. 3, 4, 5*)

Since Cable and Hunninen have described this cercaria well, only additions will be given here. *Cercaria nassicola* is a tiny xiphidiocercaria of the ubiquita group, averaging 0.2 in length. It is a slow but continuous swimmer when above the bottom; as soon as it touches the bottom, however, it moves actively over it. Touching the cercaria while it was swimming did not cause cessation of motion. No ventral sucker as such has been observed. In sectioned specimens, however, a small vacuole or cavity may be found in the region of the future suckers. Likewise, in some flattened specimens, a thickening in this region may be found. The number of glands and arrangement of ducts were found to be as described. Four flame cells were found on each side, in the formula $2[(1+1)+(1+1)]$. The stylet is quite large in comparison with the size of the oral sucker, measuring 0.023 in length. It is lancet-shaped anteriorly, bluntly rounded posteriorly, while an elongate keel protrudes a short distance ventrally.

Metacercaria (*Figs. 6, 7*)

The metacercariae occur free in the pericardial cavity of the crustacean. These roll out when the host is torn open, with no host tissue attached. In the smaller cysts, the stylet remains in position. As the metacercaria grows, the stylet is absorbed, not shed, for it was never found either in position or in the cyst cavity in the larger metacercariae. Remains of stylet glands were present in all larvae found. Most of the adult characters were found even in the youngest metacercariae examined: the two acetabula, the genitalia, and the convoluted walls of the intestinal crura. The cysts averaged 0.26 in diameter, never exceeding 0.29. The cyst wall averaged 0.015 in thickness. The body is covered with spines as far posteriorly as the acetabula. Large refractile glands are located beneath the cuticula of the whole body and render difficult observation of body systems, particularly the excretory system. The excretory bladder appears to be without a lumen, filled with large cells. The flame cell pattern is the same as that for the adult, $2[(2+2)+(2+2)]$.

The younger metacercariae are removed easily from the cyst but die very quickly. Older specimens, however, are removed with difficulty due to increased thickness of the cyst wall. These may be mounted in mammalian Ringer's solution and remain alive for several hours.

Adult (Figs. 8, 9)

Since the adult of this species has been adequately described in a previous paper (Rankin, 1939) only a few controversial details will be discussed. Yamaguti (1934, 1940) considered the second ventral sucker to be a female genital sucker located at the external opening of the uterus. In the present study, however, metacercariae of various ages, ranging from stages with only poorly developed to those with fully developed suckers, have been examined both alive and sectioned. In no case was any connection ever found between the uterus and this second sucker. In development and histological appearance it has every character of a true sucker. Figure 9 indicates the appearance of the two ventral suckers and their striking similarity is readily apparent. Perhaps phylogenetically this second sucker may have been derived from a genital sucker, having lost this former function and connection secondarily. However, the present evidence does not permit such a conclusion, but does indicate the acetabular nature of this structure.

A receptaculum seminis and Laurer's canal have been reported by Yamaguti and others as absent from genera considered here as in the family Microphallidae. The writer (1939, etc.), Cable and Hunninen (1940), and Hadley and Castle (1940) have shown that these structures do exist in species of *Levinseniella, Spelotrema, Maritrema,* and *Gynaecotyla.* A careful study of live material will probably show these structures to occur in other genera likewise, for only on living specimens have they been observed. They are so thin-walled that they collapse immediately on fixation. In *G. nassicola,* the receptaculum seminis and Laurer's canal may be discerned particularly well in immature specimens in which the uterine coils are not yet filled with eggs (Fig. 8).

The Egg and Miracidium (Fig. 10)

The structure and development of the miracidium are very difficult to follow due to its small size. The eggs measure 0.02×0.01 and are very numerous. In young worms the eggs are practically undeveloped;

EXPLANATION OF PLATE II

FIG. 6. Metacercaria within cyst. Note that even through the cyst wall the two acetabula may be observed.

FIG. 7. Metacercaria removed from cyst wall. Stylet still present along with remains of the cephalic or stylet glands; genitalia fairly well developed; flame cell pattern shown.

FIG. 8. Immature adult recovered from an experimentally infected gull. Remains of gland ducts still present. Note especially the two large, similar acetabula, and the large bulbous receptaculum seminis with a short Laurer's canal.

FIG. 9. Cross-section through the ventral suckers and the copulatory complex. The identities of the two suckers are easily discerned.

FIG. 10. Immature egg.

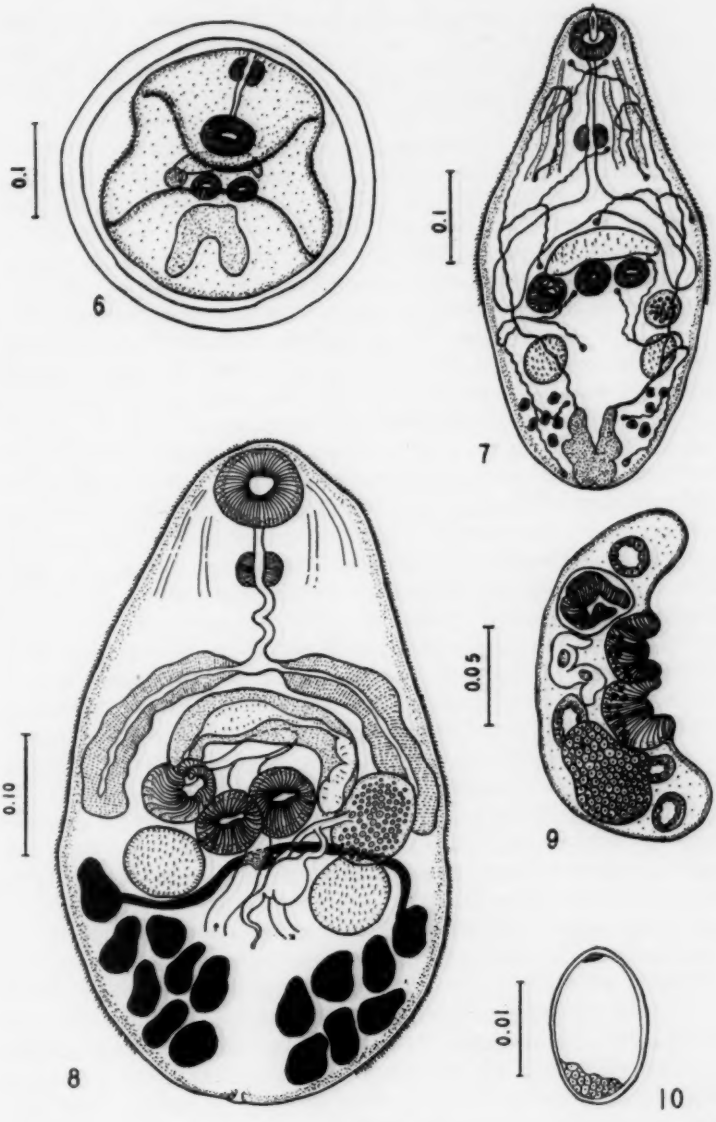


PLATE II

in older specimens, crowding prevents clear observation. Development in sea water or in mammalian Ringer's solution is very slow and no larva has been carried through to hatching. Consequently, the mode of penetration into the snail host has not been observed.

Ecology of Gynaecotyla nassicola

The relationship between the habits of host and parasite is well illustrated in the life cycle of *Gynaecotyla nassicola*. In a comparatively small area all three hosts occur in relative abundance. *Nassa obsoleta* literally covers the beach at the infected locality near the low tide zone. As the water recedes at low tide, the snails are exposed and crawl about on the sand or burrow beneath the surface. The crustaceans, likewise, emerge by the thousand from the sand at low tide and have been seen running across the damp surface in hordes. Plovers, sandpipers, and other shore birds may be observed running up and down the beach feeding on these amphipods. Other species of crustaceans usually remain buried in the sand or under drift weed along the water's edge. *Talorchestia* is the only one exposed so plentifully to the birds.

A study of the seasonal distribution of infection of *Nassa* with the cercariae of *G. nassicola* yielded the following results (the first figure after the date is the number of snails examined; the second figure is the percentage of infection): July, 1939: 717, 2.9; August: 481, 0; September: 815, 0; March, 1940: 525, 9.2; April: 576, 21.9; May: 611, 34.8; June: 1570, 6.1; July: 2640, 0.01; August: 2000, 0; September: 1250, 0. Examination of these figures indicates a relatively high snail infection in spring and little or no infection in fall and winter. A similar study of *Talorchestia* indicates little infection with metacercariae in early spring, but an increase to a maximum in late July and August.

These results might be interpreted as follows: When the birds arrive early in February and March during their northward migratory period, eggs of the parasite are dropped in the feces along the beach as the birds feed at low tide. Miracidia hatch, penetrate *Nassa obsoleta,* and develop into sporocysts. The birds stay at Woods Hole only for a few days at this time and then resume their northward trek to the breeding grounds. When they return in the fall, the cercariae have matured, penetrated the beach fleas, and developd into ripe metacercariae. The birds, then, may become reinfected and carry the worms back on their southward migration. Possibly the snails might become reinfected at this time also, the developing larvae remaining dormant through the winter. But since little or no infection was found in early spring, this hypothesis does not seem well grounded.

Discussion and Conclusions

Until the life cycles of *Gynaecotyla nassicola, Maritrema arenaria* (Hadley and Castle, 1940), and *Spelotrema nicolli* (Cable and Hunninen, 1940) were described, an understanding of the relationships of the Microphallidae was handicapped considerably. The genera of this family were included with the Heterophyidae on purely superficial morphological characters. Ward (1901) erected the subfamily Microphallinae to contain these genera, but he still retained them under the Heterophyidae. Travassos (1921) raised the Microphallinae to family rank, excluding it from the Heterophyidae, as did Viana (1924). Witenberg (1929) likewise excluded the Microphallinae from the Heterophyidae. In spite of these observations, various investigators have retained the microphallids under the Heterophyidae. It seems pertinent at this point, therefore, to indicate briefly the main differences between these two families of trematodes. In the Microphallidae, the uterus lies either wholly posterior to or overlapping the testes; there is a well-developed acetabulum, not enclosed with the genital suckers; the genital pore is not closely associated with the acetabulum; the excretory system is always of the formula $2[(2+2)+(2+2)]$; and the life cycle always includes a crustacean as the second intermediate host. In the Heterophyidae, the uterus lies anterior to the testes; the acetabulum is usually closely associated with the genital structures; the excretory system is never of the above pattern, but consists of large numbers of flame cells on each side of the body; and the life cycle usually includes a fish as the second intermediate host. In the first family, a crustacean-eating animal (usually a bird) is the definitive host, whereas in the second family a fish-eating animal (mammal or bird) is the definitive host. These characters, along with other morphological features, render impossible the inclusion of members of one with those of the other. As pointed out by the writer (1939*c*) and Cable and Hunninen (1940), the complete life cycle of a species should be known before final conclusions can be drawn with respect to relationships, distribution, etc.

The infection of second intermediate hosts through the respiratory system by both *Gynaecotyla nassicola* and *Spelotrema nicolli* would indicate that other members of this family infect the hosts through this system rather than through the digestive tract. Depending on the type of respiratory apparatus of the particular crustacean, the cercariae are either swept in passively through a gill chamber and then penetrate, or actively bore into branchial lamellae.

Considering the results obtained from a seasonal-distribution study of the northward range of the cycle, it would prove of considerable in-

terest to examine the different hosts at the southern range. A similar distribution might occur. If this were so, then added evidence for the hypothesis that the northern snails do not remain infected during the winter would be obtained.

A specificity for hosts is evidenced to a marked degree by the different stages of *G. nassicola*. *Nassa obsoleta* and *Talorchestia longicornis* are the only intermediate hosts found infected or infectable, of the many examined. The distribution of these hosts in the vicinity of the definitive host may account for this phenomenon. Should the habits of other mollusks and crustaceans bring them in proximity to the birds, then they in turn might be expected to become infected. Yet, since no other species could be experimentally infected, the ecological factor is not the determining one. Some physiological difference seems evident. Host preference has been reported by various writers (Lühe, 1909; Dubois, 1929; Wesenberg-Lund, 1934; Rankin, 1939*c*; etc.) and seems to be a fairly common phenomenon.

Summary

The life cycle of the microphallid trematode, *Gynaecotyla nassicola* (Cable and Hunninen, 1938) Yamaguti, 1939, has been determined experimentally and the various stages described and figured. The miricidia penetrate the mud snail, *Nassa obsoleta* (Say), develop into oval sporocysts that produce daughter sporocysts and cercariae of the ubiquita type. The cercariae penetrate the branchial lamellae of the sand flea, *Talorchestia longicornis* (Say), then migrate through the tissues to the pericardial cavity where they encyst. Metacercariae develop into adults when the crustaceans are eaten by shore birds (plovers, sandpipers, etc.) or fed to experimental hosts (gulls).

The ecology of the hosts and parasite is discussed, indicating the close relationship between the distribution of the various animals involved. The differences between the Heterophyidae and the Microphallidae are indicated.

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NITROGEN CONTENT AND DISTRIBUTION IN EGGS OF MELANOPLUS DIFFERENTIALIS DURING EMBRYONIC DEVELOPMENT[1]

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Introduction

During the past few years, attention has been given to studies on the physiology of the egg and embryo of the grasshopper, *Melanoplus differentialis*. Since most of this work has been based on the intact egg as a unit, a growth curve based on the total nitrogen as an index of the protein content seems desirable as a further basis of reference. A study has therefore been made to determine the nitrogen content of eggs of various ages and also the distribution of this nitrogen among shell, yolk and embryo.

Materials and Methods

The preparation of the eggs consisted in removing them from the pods in which they were laid and washing in distilled water to remove any adhering substances. They were kept at 25° C. until they had reached the desired stage or until they had entered the diapause, or blocked state (Slifer, 1932). In order to obtain postdiapause eggs of a known developmental stage, the eggs were subjected to cold (5° C.) soon after diapause began and kept at that temperature for several months. When the temperature is again raised to 25° C., they resume development and hatch in 18 or 19 days. Before analysis, samples of the eggs were examined after dissection to make sure that all were in the desired developmental stage.

For the first part of the study, the original Kjeldahl method for total nitrogen determination as outlined in Hawk and Bergeim's "Practical Physiological Chemistry" (but using a smaller apparatus) was used on lots of about 50 diapause eggs. Some of the lots contained eggs from one pod only, while others were obtained by mixing eggs from 3, 4, or 5 pods. These mixtures gave a value nearer the average, as the eggs of one pod tend to vary less widely than eggs from different pods.

The reagents used were a digestion mixture of 3 grams Cu_2SO_4 and

[1] Aided by a grant from the Rockefeller Foundation for work on the physiology of the normal cell.

1 gram K_2SO_4 in 300 cc. concentrated H_2SO_4; concentrated NaOH solution, for neutralization; 0.0706 N HCl (1 cc. ≡ .9886 mg. N), for receiving the NH_3; and 0.1074 N NaOH, for back titration. The amounts used were about 15 cc. of the digestion fluid, sufficient NaOH to provide an excess of alkali after neutralization of the acid, and approximately 10 cc. of HCl (added from a stopcock burette). Back titration was done with a 50 cc. Mohr burette and with phenolphthalein as an indicator.

The larger part of the work was done by means of Keys' modification of the Kjeldahl technique for rapid microanalysis (Keys, 1940). With this modification, quantities of 0.01–20.0 mg. of total nitrogen per sample may be analyzed. The digestion was carried out by a mixture of Cu_2SO_4, K_2SO_4, and H_2SO_4 as in the previous Kjeldahl method and phenolphthalein was used as an indicator. These are not the reagents used by Keys, but the change does not appreciably affect the accuracy of the method. The HCl was measured into the receiving tube from a .2 cc. pipette graduated in hundredths and .2, .3, or .4 cc. of the acid were used depending upon the sample to be analyzed. The acid was the same as in the previous method (1 cc. ≡ .9886 mg. N) but the NaOH was 0.10025 N. One to 5 cc. of the digestion mixture were required. Titration was done by means of a burette made from a similar .2 cc. pipette fixed with a 1 cc. syringe according to the principle of the Linderstrøm-Lang pipette (Glick, 1935). It can be easily controlled and read to one thousandths of a cc.

Single eggs were analyzed and in addition determinations were run on embryos, shell and yolk separately. The embryos were dissected out and washed, the shells washed, and the washings and yolk combined. Each part was then analyzed separately. For the earlier stages 2 embryos were analyzed since one contains too little nitrogen for accurate determination. A complete series of stages both before and after diapause were analyzed by this procedure. Until the seventh day after laying, the embryos are too small to handle, so no determinations were possible on these younger stages. In late postdiapause eggs, after the yolk mass was completely engulfed by the embryo (about 7 days postdiapause), the unabsorbed yolk was pressed out of the embryo and analyzed separately although some workers include it with the embryo. In all cases, 1-cc. samples of a known solution (1 mg. NH_4Cl in 10 cc.) were analyzed after every 3 to 5 determinations as a check on the reagents and apparatus. If the error exceeded 2 per cent, the determinations done just previously were discarded.

Accuracy

Macrodeterminations were used only when the results on known samples were within 5 per cent of the known value. This error is rather large, but even using 50 eggs the quantity of nitrogen is close to the lower

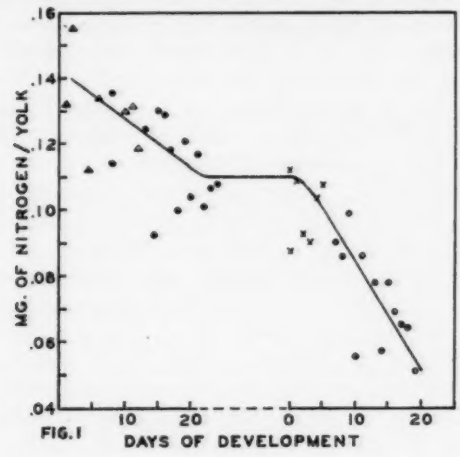

FIG. 1

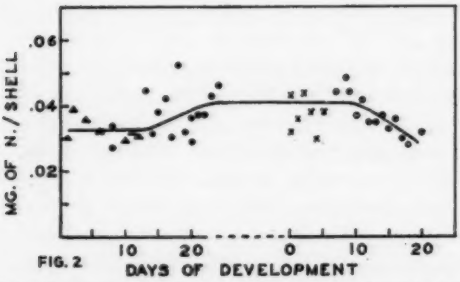

FIG. 2

Fig. 1. Yolk nitrogen. Each point indicates one determination and each pod is represented by a different symbol. The diapause period, which lasts for several months, is shown by the dotted portion of the abscissa. The level of the curve during this period was determined from an average of several analyses.

Fig. 2. Shell nitrogen. The symbols are the same as in Fig. 1.

limit for accurate determination by this method, and too many eggs would have been required to obtain greater accuracy. However, variations between lots from different pods were as much as 20 per cent so that the error in determination is not too great for statistical study.

All determinations of known solutions done by microanalysis were

within 2 per cent of the theoretical value or the experimental analyses just preceding were discarded. The control values ranged between 101.8 per cent and 98.1 per cent but 99.8 per cent and 100.8 per cent were the values more often found. These values correspond to burette readings of 69 and 70 mm.[3] while the extremes used were 68 and 71. The 2 per cent accuracy is all that is necessary since individual variation may be as much as 20 per cent between diapause eggs from different pods.

Results

Macrodeterminations were used to establish the value for the total nitrogen of the intact diapause egg. The eggs are quite variable and therefore a wide range of nitrogen values even for eggs from the same pod exists. The variation is even greater when different pods are used. For this reason, it was thought advisable to use the results of the macro-

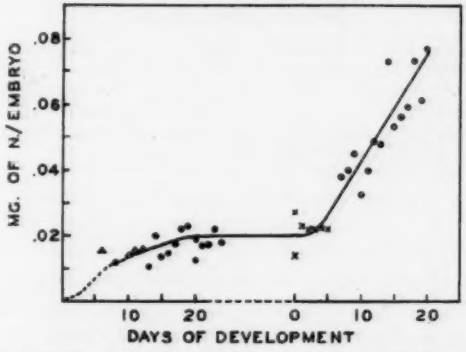

Fig. 3. Embryo nitrogen. Symbols as in the preceding graphs.

analyses to determine the statistical average. The value assigned (174 γ) for the diapause is the average of 18 determinations of 50 eggs each. This serves as a check on the other curves made from micro-determinations on fewer pods.

It will be seen from the yolk curve (Fig. 1) that a wide variation in the yolk nitrogen accounts for most of the variation of the egg as a whole. This can be seen by comparing the individual points on the graph of the yolk (Fig. 1) with those on the curve plotted by adding together the 3 parts of the egg (Fig. 4). In spite of the rather wide variation, a definite trend downward may be seen. The yolk nitrogen drops from about 80 per cent of the total nitrogen at laying to about 60 per cent at diapause. During diapause it remains constant and when development is resumed it decreases rapidly from 110 γ at diapause to 50 γ at hatching.

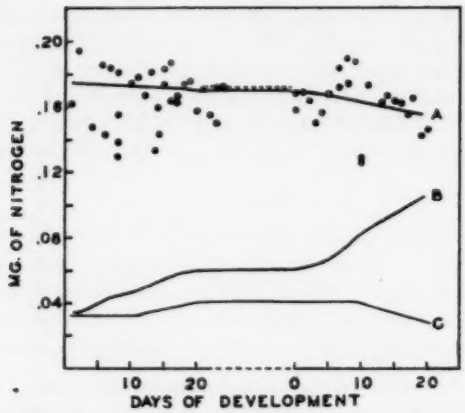

FIG. 4. A composite of the three preceding graphs No individual determinations were used but the three smoothed curves were added. *C*, shell *N*; *B*, shell and embryo *N*; *A*, shell plus embryo plus yolk *N*. The ordinate length beneath curve *C* is a representation of the shell nitrogen; that between *B* and *C*, the embryo nitrogen; and between *A* and *B*, the yolk nitrogen. The level of the broken line is the level for diapause determined by the macromethod.

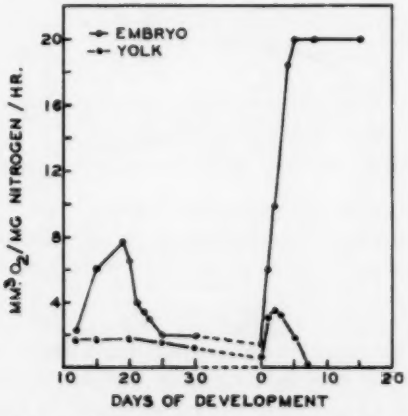

FIG. 5. Curves obtained by dividing the O_2 consumption curves of Boell and Bodine (1936) by the embryo and yolk *N* curves of this paper, showing the changes in basal respiration, i.e., mm.^{3} O_2/mg. nitrogen/hr., during embryonic development.

The shell nitrogen (Fig. 2) was found to be about 30 γ per egg until about the tenth day. Between the tenth and twentieth days it rose to more than 40 γ per egg, which was maintained during the diapause period. Between 5 days postdiapause and hatching, the shell nitrogen decreased to about 35 γ.

The curve for embryo nitrogen (Fig. 3) rises steadily during prediapause development, is level at diapause, and again rises rapidly in the postdiapause period.

Figure 4 is a composite of the three preceding graphs. The points plotted for the nitrogen of the whole egg were found by analyzing whole eggs (solid symbols) and by adding the values found for embryo, shell, and yolk of the same eggs (open symbols). These points are extremely scattered and no curve can be drawn accurately based on these points. The curve drawn was made by adding the smoothed curves of the separate parts. The diapause level thus determined is not significantly different from the level taken from the average of the macrodeterminations on diapause eggs.

Discussion

The curve (Fig. 4) for the nitrogen of the whole egg is too indeterminate for any accurate quantitative analyses of the nitrogen behavior but it can be seen by the addition curve that no significant change, either increase or decrease, in nitrogen during the embryonic period occurs. The organism seems incapable of utilizing atmospheric nitrogen.

The early rise in shell nitrogen, as well as the drop in yolk nitrogen, corresponds to the formation of the white cuticle which begins at about the tenth day (Slifer, 1937). The loss of nitrogen from the shell in the postdiapause period is doubtless due to the digestion of this layer preparatory to hatching (Slifer, 1937).

The embryo nitrogen curve is comparable in shape to curves for various physiological activities of the embryo. A $mm.^3$ O_2/mg. embryonic N/hr. curve has been made (Fig. 5) by dividing the $mm.^3$/embryo/hr. curve of Bodine and Boell (1936) by the mg. N/embryo curve of this paper, which may be used as a basal curve for future work. The rate of O_2 consumption per mg. of embryo nitrogen rises steadily in prediapause, but drops rapidly at the onset of diapause, the diapause level being 1.7 $mm.^3$ O_2/mg. N. As soon as development resumes the rate rises again, until, on the fifth day, it reaches 20 $mm.^3$ O_2/mg. N where it remains practically constant throughout the remainder of the embryonic period.

During prediapause the O_2 consumption of the yolk (Fig. 5) is a constant value, 1.8 $mm.^3$ O_2/mg. N. It drops during diapause to .5

mm.^{3} In the postdiapause period, it rises to a peak of 3.7 mm.^{3} in 2 days and then drops rapidly to zero when the yolk has been engulfed by the embryo.

Summary

The total nitrogen content of the egg of the grasshopper, *Melanoplus differentialis,* has been determined and also the changes in its distribution among shell, embryo, and yolk. The nitrogen content of the whole egg is constant while the embryo nitrogen increases at the expense of the yolk nitrogen. The changes in shell nitrogen correspond to the formation and digestion of the cuticle. No change occurs during diapause.

A basal metabolism curve is computed from the embryo nitrogen curve of this paper and the oxygen consumption curve of Boell and Bodine (1936) which shows that the oxygen consumption rate rises to 20 mm.^{3} O_2/mg. N at 5 days postdiapause and remains steady at that value. A similar curve is computed for the yolk. The metabolic rate is constant in the prediapause at a value of 1.8 mm.^{3} O_2/mg. N and in the postdiapause drops from a peak at 2 days to 0 at 10 days. Both curves show very low rates during diapause.

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FACTORS DETERMINING THE DISTRIBUTION OF POPULATIONS OF CHAETOGNATHS IN THE GULF OF MAINE[1]

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In aquatic environments, and particularly in large bodies of water, the movement of the water itself may profoundly affect both the distribution and numbers of the population. Pelagic organisms are carried with the water in its drift through each given region. Numerical abundance may thus depend on conditions in external regions from which the water comes. The moving water affects the climate of the region into which it flows, for it carries with it its inherent temperature, salinity, etc. At the same time these properties of the water may be profoundly altered in its course, through interaction with the atmosphere and with other bodies of water. The maintenance of a population in a given region under these conditions depends upon a balance of dynamic factors; the drift of the water and its interaction with its environment, as well as upon the rate of reproduction and mortality of the population under the environmental conditions determined by these circumstances (Russell, 1936). The pelagic population is at the mercy of the hydrodynamic factors, since it must move with the water. It is only by taking advantage of fortuitous hydrographic conditions, such as the existence of permanent eddies and dead waters, that they may gain a truly endemic relation to a given region.

The use of plankton as indicators of water movements has attracted deserved attention of late (Russell, 1936*a*, 1939). The effect of the circulation of water on the distribution and maintenance of permanent populations of specific organisms has received less explicit attention, but is exemplified by the studies of Damas (1905), Sømme (1933, 1934) and Redfield (1939).

As Huntsman (1919) has shown, the chaetognaths are an unusually interesting group for studies in oceanic zoögeography. Of the half-dozen species found in the coastal waters between Cape Cod and the Grand Banks, three are of deep-water origin and are drawn into the deeper basins of the Gulf as an immigrant population which perishes without reproducing. Three other species are inhabitants of the upper layers of the sea, of which one breeds endemically on the continental

[1] Contribution No. 279 from the Woods Hole Oceanographic Institution.

shelf, while the other two are immigrants from the oceanic waters offshore. These six species must have essentially similar habits of life, yet each has a characteristically different distribution, depending upon the water body in which it maintains itself endemically, and upon the degree to which these waters are drawn into the various regions of the coast. The value of Sagittae as indicators of water movements in British waters has been demonstrated by Russell (1935, 1936*b*), Fraser (1937, 1939) and Pierce and Orton (1939).

The present paper is an examination of the populations of chaetognaths found in the Gulf of Maine during the year 1933–34, when the research vessel of the Woods Hole Oceanographic Institution, the "Atlantis," made a systematic survey of the region. While the results confirm in the main the observations of Bigelow (1926) in this region and

TABLE I

Statistics of collections of chaetognaths in the Gulf of Maine, 1933–1934.

Dates of Cruise	Number of Stations at Which Chaetognaths were Present						
	S. elegans	*S. serratodentata*	*S. maxima*	*S. lyra*	*S. enflata*	*E. hamata*	Total Stations
Sept. 2–14, 1933	30	12	6	2	1	14	35
Dec. 2–11, 1933	17	9	5	1	0	13	22
Jan. 3–13, 1934	11	7	2	2	0	9	12
Mar. 21–28, 1934	10	8	0	0	0	15	18
April 17–May 13	20	9	2	0	0	16	32
May 21–June 2	32	20	4	1	0	21	44
June 25–July 1	6	2	0	0	0	2	7
Sept. 17–24, 1934	20	16	4	3	1	15	25
Total	146	83	23	9	2	105	195
Per cent present	75	43	12	46	1	54	

Dates of Cruise	Numbers of Chaetognaths Taken at These Stations						
	S. elegans	*S. serratodentata*	*S. maxima*	*S. lyra*	*S. enflata*	*E. hamata*	Total
Sept. 2–14, 1933	2,961	104	9	4	2	107	
Dec. 2–11, 1933	3,608	45	17	1	0	300	
Jan. 3–13, 1934	194	8	3	3	0	87	
Mar. 21–28, 1934	105	13	0	0	0	95	
April 17–May 13	785	137	8	0	0	274	
May 21–June 2	1,698	104	21	2	0	289	
June 25–July 1	368	33	0	0	0	2	
Sept. 17–24, 1934	453	534	5	3	5	133	
Total	10,170	978	63	13	7	1,287	12,418
Per cent of total	82	7.9	0.51	0.10	0.06	9.5	—

Dates of Cruise	Mean Catch per Haul at Stations at Which the Species was Present						
	S. elegans	*S. serratodentata*	*S. maxima*	*S. lyra*	*S. enflata*	*E. hamata*	Total
Sept. 2–14, 1933	98.7	8.7	1.3	2.0	2.0	7.6	
Dec. 2–11, 1933	212.0	5.0	3.4	1.0		22.1	
Jan. 3–13, 1934	17.6	1.1	1.5	1.5		9.7	
Mar. 21–28, 1934	10.5	1.6	—	—		6.3	
April 17–May 13	39.1	15.2	4.0	—		17.0	
May 21–June 2	53.0	5.2	5.3	2.0		13.7	
June 25–July 1	61.5	16.5	—	—		1.0	
Sept. 17–24, 1934	22.7	33.4	1.25	1.0	5.0	8.9	
Mean catch per haul for entire period	69.8	11.8	2.74	1.44	3.5	11.3	

Dates of Cruise	Per Cent of Total Hauls in Each Period at Which Each Species was Present						
	S. elegans	*S. serratodentata*	*S. maxima*	*S. lyra*	*S. enflata*	*E. hamata*	Total
Sept. 2–14, 1933	86	34	17	5.7	2.9	40	
Dec. 2–11, 1933	77	41	23	4.5		59	
Jan. 3–13, 1934	92	58	17	17		75	
Mar. 21–28, 1934	56	45	0	0		83	
April 17–May 13	63	28	6.3	0		50	
May 21–June 2	73	45	9	2.3		48	
June 25–July 1	86	29	0	0		14	
Sept. 17–24, 1934	80	64	16	12	16	60	
Entire period	75	43	12	4.6	1	54	

of Huntsman (1919) in Canadian waters, it has seemed worthwhile to examine them together with those of Bigelow and Huntsman from the point of view outlined above.

Material and Data

General Statistics

The cruises were planned to include lines of stations extending from shoal water across the Gulf so as to sample all the principal parts of the Gulf and the enclosing banks. While exigencies of weather and the loss of nets interfered with the completeness of the program, the 195 hauls included in this study amount to a fairly satisfactory random sampling of the region, throughout the year.

The collections were made with a standard 1.5 meter Heligoland larva net (No. 0 silk, 38 meshes to the inch) drawn vertically from a point near the bottom to the surface. The opening of the net had an area of 1.7 square meters. Table I contains some statistical data on the collections.

Numerical Abundance.—The 12,418 specimens taken give a mean catch of 64 per haul or 37.5 per square meter of sea surface. Of these by far the most abundant was *S. elegans,* amounting to 82 per cent of the total. With *S. serratodentata* yielding 7.9 per cent, these two shallow-water forms accounted for 90 per cent of the total population. Of the three deep-water species *E. hamata* accounted for 9.5 per cent of the catch, with *S. maxima* and *S. lyra* making up 0.5 and 0.1 per cent respectively.

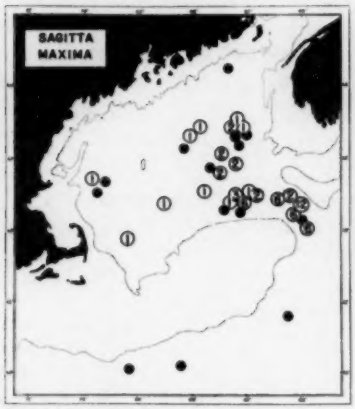

FIG. 1

FIG. 2

FIG. 1. Locations at which *Sagitta maxima* has been taken in the Gulf of Maine.

FIG. 2. Locations at which *Sagitta lyra* has been taken in Gulf of Maine. Figures in circles indicate the numbers taken per haul from "Atlantis" in 1933–34. Solid circles indicate Bigelow's captures.

The relative breadth of distribution of the species is indicated by the percentage of stations at which each was taken. The general order is the same as for the numerical abundance. The species differ, however, far less in distribution than in numerical abundance, e.g., *S. serratodentata* and *E. hamata* which accounted for only 8 and 9 per cent of the total numbers but occurred at more than 40 and 50 per cent of the stations.

Numerical abundance depends on both the extent of distribution and the density of population within that distribution. A general idea of the latter is given by the mean size of the catch at each station at which a given species is present. Again the order is essentially the same, *S. elegans* leading with a mean density of 70 per haul, *S. serratodentata* and *E. hamata* following with 12 and 11 per haul, while *S. maxima* and *S. lyra* occur sparsely when at all.

Seasonal Fluctuations

Sagitta elegans and *S. serratodentata* show a decline in total numbers taken, and in the mean density of the population at the stations where they were present during the colder part of the year. The figures are irregular as both forms occur occasionally in dense swarms, but it appears that these species are reduced to perhaps one-tenth their numbers during the winter. The cruises in December, January and March did not sample adequately the waters of Georges Bank where the larger catches of *S. elegans* were taken at other seasons and this may exaggerate the apparent effect of winter on this species, although it can scarcely do so in the case of *S. serratodentata,* which occurs chiefly over the deeper part of the basin which was adequately sampled.

Contrasted with these inhabitants of the upper levels, in which the seasonal change in temperature is great, the deep-water species *E. hamata* shows no systematic fluctuation either in total numbers, the percentage of stations at which it is present, or numbers taken per haul, during the year. Bigelow (1926) has pointed out that this is to be expected since the depths at which it lives are not subject to much change in temperature.[2]

The Distribution of the Deep-Water Species

Sagitta maxima, S. lyra and *Eukrohnia hamata* are inhabitants of the deeper waters of the Gulf of Maine. Figures 1 and 2 record the location and numbers of the two former species taken in each haul while Fig. 3 shows the general pattern of distribution of the more abundant *E. hamata.* All our specimens of *S. maxima* were taken in water of over 170 meters depth and it occurred in greatest abundance in depths of over 200 meters. It seems safe to conclude with Huntsman and Bigelow that it occurs only in the deeper parts of the Gulf, chiefly below the 150-meter level. The distribution of the catches of *S. lyra* is essentially the same as that of *S. maxima.* The regional occurrence of *E. hamata* is similar to that of the deep-water sagittae, though it was taken more frequently in the shoaler regions along the margins of the Gulf, occasionally in water of as little as 100 meters depth. It appears to occur rather nearer the surface than do the deep-water sagittae.

The distribution of catches of the three species agree in showing the highest frequency of successful catches and the greatest numbers per haul in the Eastern Channel through which deep water has access to the Gulf. Inside the Gulf the populations are concentrated in the eastern

[2] Bigelow considered that *S. maxima* occurred in the Gulf more frequently in winter, *S. lyra* in summer. Our data do not confirm this. Their numerical abundance is rather closely correlated throughout the year and neither shows a clear seasonal variation.

basin, and diminish in abundance northward and westward as the Eastern Channel becomes remote.

Dynamic contours give the best available information concerning the probable circulation of the deeper water. Contours representing the circulation at 140 meters in May–June, 1934 are indicated in Fig. 5.[3] They represent a pattern which occurs with some variation in detail from month to month at this and greater depths. The center of abundance of the three deep-water chaetognaths lies in the rapidly moving inflow or in the eastern side of the eddy which has its vortex in the deep basin northwest of the Eastern Channel. Once the creatures pass beyond the vortex they become scattered and decrease in numbers.

The distribution of the deep-water chaetognaths suggests strongly that they are carried into the Gulf from offshore, along with the warm

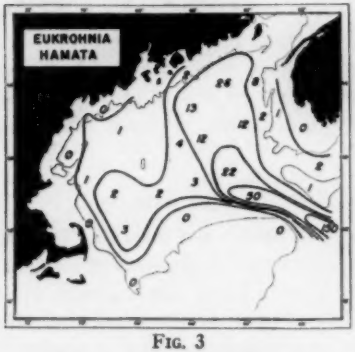

FIG. 3

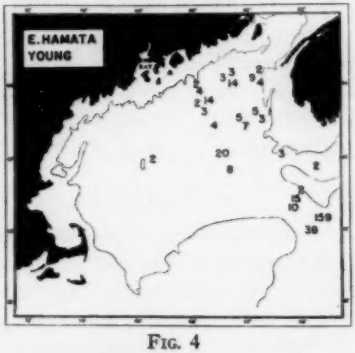

FIG. 4

FIG. 3. Relative abundance of *Eukrohnia hamata* in different parts of the Gulf of Maine. Numbers represent the average catch per haul in various regions.

FIG. 4. Locations at which young specimens of *Eukrohnia hamata* were taken in 1933–34. Numbers indicate the number of young taken per haul at each position.

saline water which Bigelow (1927) found to enter through the Eastern Channel. Since there is no evidence that they breed within the Gulf, they are to be considered as terminal immigrants from other regions in which they breed endemically. While some of those which are carried into the Gulf may complete the circuit of the eddy and be carried out again, the greater number are probably trapped within the dead waters of the inner Gulf, live as long as circumstances permit and die without leaving progeny.

The foregoing observations and conclusions are in entire agreement with those of Bigelow (1926). In certain details, however, our findings

[3] A hydrodynamic analysis of the circulation of the Gulf of Maine during the period of our survey is being prepared by Dr. E. E. Watson, to whom I am indebted for the use of this and subsequent figures.

differ from his. While he considered *E. hamata* to occur with regularity throughout the year, the sagittae were thought to fluctuate with the season, *S. maxima* being taken predominantly in the colder part of the year and *S. lyra* in the warmer. He proposed that *S. maxima,* an inhabitant of northern waters, is barred from the Gulf by the warmth of the water entering the Eastern Channel during the summer months. The statistics recorded in Table I fail to show a clear fluctuation in any of the deep-water species which may be correlated with the season. That *S. maxima* may enter the Gulf at a time when the water is warmest is evidenced by our taking 12 and 6 specimens in two hauls made in the Eastern Channel in May when the temperature of the deeper water was 8°–9° C. and 2 in a September haul from water varying in temperature from 6.5° to 8.76°.

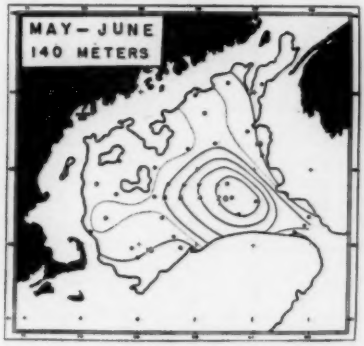

FIG. 5

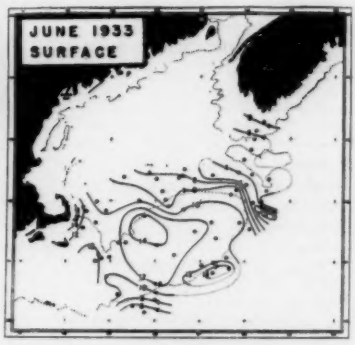

FIG. 6

FIG. 5. Dynamic contours indicating theoretical circulation in the basin of the Gulf of Maine at depth of 140 meters in May–June, 1934.

FIG. 6. Dynamic contours indicating theoretical circulation at surface of Georges Bank in June, 1933.

We believe he was misled, particularly in the case of *S. lyra,* of which he took only 6 examples, by the fact that his cruises did not sample the center of abundance of the populations as thoroughly as the more remote and colder periphery of the range.

Factors Limiting the Distribution within the Gulf

In attempting to evaluate the relative importance of the circulation of the water and of environmental factors in determining the abundance and distribution of the species, it is crucial to inquire whether the numerical relations are due to the degree to which the three species are "adapted" to the conditions in the Gulf, or whether they are to be explained by the relative numbers in which they enter from offshore.

In the former case the numerical ratios should shift as one compares waters increasingly remote from the point of entrance. In the latter they should remain unchanged.

To examine these possibilities, we have divided the catches in which any of the species occurred into three groups: (1) those taken in the deep water of the Eastern Channel (east of 66°30′ and south of 42° 40′N); (2) those lying immediately within the basin (a quadrangle lying between 42°10′ and 43°30′N and 66°30′ and 67°30′W), and (3)

TABLE II

Relative numbers of deep-water chaetognaths taken in different areas in Gulf of Maine.

Total Numbers	*E. hamata*	*S. maxima*	*S. lyra*
Eastern Channel	434	34	2
Southeastern quadrangle of basin	411	25	6
Remainder of basin	442	4	5
Total area	1287	63	13
Relative Numbers Taken			
Eastern Channel	100	7.8	0.5
Southeastern quadrangle of basin	100	6.1	1.5
Remainder of basin	100	0.9	1.1
Total area	100	5.0	1.0
Stations Present			
Eastern Channel	9	6	1
Southeastern quadrangle of basin	19	11	3
Remainder of basin	87	5	5
Total area	105	23	9
Relative Numbers of Stations Present			
Eastern Channel	100	67	11
Southeastern quadrangle of basin	100	58	5.7
Remainder of basin	100	5.7	5.7
Total area	100	22	8.6

the remainder of the Gulf. These three areas yielded *E. hamata* in about equal numbers. They differ in proximity to the external sources of the deep water of the Gulf.

The numbers of specimens and the relative numbers of each species taken in these areas are given in Table II. They show that a great disparity between the abundance of the three species already occurs in the water entering the Channel. In the main the relative abundance is established by conditions external to the Gulf, and cannot be attributed merely to the selective effect of conditions within.

There is, however, clear evidence that some selective mechanism is at work limiting the penetration of *S. maxima* into the remoter region. The ratio in which *S. maxima* is taken in proportion to the catch of *E. hamata* is eight times as great in the Eastern Channel as in the remoter region. The distribution of *S. lyra* appears to resemble that of *E. hamata* more closely than that of *S. maxima*.

TABLE III

Catches of Sagitta maxima and Sagitta lyra classified according to highest temperature of the water column (below 100 meters) from which they were taken.

Temperature, ° C.	*S. maxima*							
	5–5.5	5.5–6.0	6.0–6.5	6.5–7.0	7.0–7.5	7.5–8.0	8.0–8.5	8.5–9.0
Number per haul	1	1 1	2	3	2 2 1 1 1	6 2 1	12 6 6 3 2 1	6 2
Total	1	2	2	3	7	9	30	8
Per cent of total	1.59	3.18	3.18	4.76	12.7	14.3	47.7	12.7
Integrated per cent	1.59	4.77	7.95	12.71	25.4	39.7	87.4	100
Mean catch per haul	1	1	2	1.5	1.3	3	5	4
Temperature, ° C.	*S. lyra*							
	5–5.5	5.5–6.0	6.0–6.5	6.5–7.0	7.0–7.5	7.5–8.0	8.0–8.5	8.5–9.0
Number per haul					1 1 1 1	1	3 2 2 1	
Total	0	0	0	0	4	1	8	
Per cent of total	0	0	0	0	30.8	7.7	61.6	
Integrated per cent	0	0	0	0	30.8	38.5	100	
Mean catch per haul	0	0	0	0	1	1	2	

In the discussion of the geographical distribution of the chaetognaths,—as of marine plankton in general,—temperature and salinity are commonly considered as factors limiting the distribution of the species. The water in which the chaetognaths of the depths appear to be carried as they penetrate the Gulf undergoes admixture with subsurface layers. As a result it becomes colder and less saline. While not wishing to suggest that these factors are not in a broad way influential in determining the survival of the species, we do wish to question whether they, or

similar environmental conditions, are essential in accounting for the differences in their distribution within the Gulf of Maine. While our data suggest that *E. hamata* invades colder water more frequently than *S. maxima* and *S. lyra,* there is no evidence that these latter cannot survive in water at least as cold as any in which we have taken the former species. Bigelow (1926, p. 325) records captures of *S. maxima* off the continental slope in waters of 3° to 6° C. and considers this species to be a distinctly cold-water form. *S. lyra* was taken by him in water of 6° C.—below which only 8 per cent of the total catch of *E. hamata* was taken. Though it may be considered a creature of relatively warm water, we have shown that it penetrates into the colder parts of the Gulf, relatively as frequently as does *E. hamata.*[4]

We believe the disparity in distribution of the deep-water chaetognaths in the Gulf can be explained more readily in another manner. We have presented in Tables III and IV data showing the incidence of

TABLE IV

Catches of Eukrohnia hamata classified according to highest temperature of the water column (below 100 meters) from which they have been taken. Total catch = 1287.

Temperature, ° C.	2–3	3–4	4–5	5–6	6–7	7–8	8–9	9–10	10–11
Total hauls.....	7	13	15	32	40	28	11	4	1
Total caught.....	7	11	33	61	213	407	310	244	1
Per cent of total....	0.54	0.86	2.56	4.74	16.5	31.5	24.0	18.9	—
Integrated per cent..	0.54	1.40	3.96	8.70	25.2	56.7	80.7	99.0	100
Mean catch per haul	1.0	0.85	2.2	1.9	5.3	14.5	28.2	60	1

the species in water of different maximal temperatures. The catches have been classified according to the temperature of the water in which they were taken. We have chosen the highest temperature recorded below 100 meters, since there is no way of knowing the level at which an animal caught in a vertical haul is taken, and since this temperature will represent the least departure from the condition under which the water entered the Gulf. The temperature profiles shown in Fig. 7 give

[4] Salinity cannot readily be separated from temperature as a possible limiting factor within the deep water of the Gulf because variations in temperature and salinity alike are due to mixtures in varying proportion of the deep, warm, and saline water with the superficial, cold, and dilute water. As a result, the temperature-salinity diagram for waters in all parts of the Gulf below the level of homogeneous winter mixing is almost a straight line and is nearly the same in each region.

It is, moreover, extremely difficult in view of what is known experimentally concerning the osmotic regulation of marine invertebrates, to believe that changes of one or two parts per mille would vitally affect animals native in 34 or 35 ‰ salinity.

some idea of the possible range of temperature in various regions of the Gulf.

Tables III and IV show the result of this classification. Though not corrected for the relative number of hauls taken in each kind of water, this correction may be neglected in comparing the different species since the data for each are derived from the same collection of catches. For each species the numbers fall off sharply as the temperature declines. In the case of *S. maxima* and *S. lyra* the great change occurs at about 7° C. *E. hamata* appears to succeed better in penetrating the colder parts of the Gulf, the numbers caught falling markedly only below 6° C.

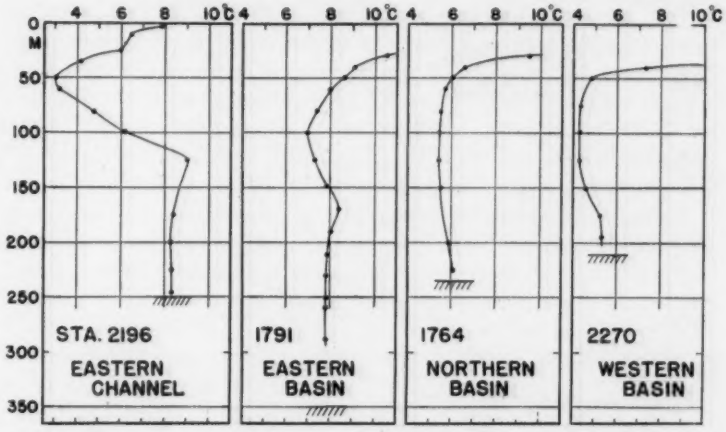

FIG. 7. Distribution of temperature with depth at a number of stations at which *Sagitta maxima* was taken.

In Fig. 8 we have plotted the integrated data showing the fraction of the total catch of each species which has penetrated to regions of decreasing temperature. The curves in Fig. 8 might be interpreted as representing the relative tolerance of individuals of the three species for the physical characteristics of the water. When the dynamic nature of the situation is considered, we believe they may be more plausibly interpreted as depending on the time of survival to be expected by the numbers which enter the Gulf, and irrespective of any change in the physical characters of the environment to be encountered therein except insofar as the conditions in the Gulf are unsuitable for its reproduction. Since the changes in the physical characteristics of the deep water depend on mixing processes, and since these must proceed relatively uniformly in all parts, the change in temperature and salinity is a function of the

time during which the water has been exposed to these processes. Temperature and salinity are thus a function of the age of the deep water in the Gulf. The abscissa of Fig. 8 may be replaced by a time scale reading from right to left and measuring in a general way the time since the water with its included population entered the Gulf.

With these considerations in mind, the curves in Fig. 8 may be interpreted as probability integrals representing the expectancy of life of the groups of chaetognaths which appear in the Eastern Channel. *E. hamata* has an expectancy of longer life than the others. Hence it has time to be carried into the remoter parts of the Gulf in large numbers

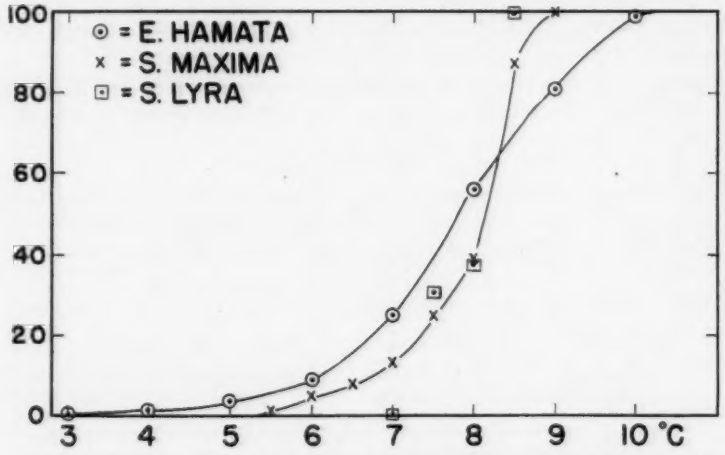

FIG. 8. Percentage of the total catch of *Eukrohnia hamata, Sagitta maxima* and *S. lyra* taken at stations at which the water below 100 meters was colder than the temperature shown on the abscissa. Based on integrated percentages shown in Tables II and III.

before it dies. Why its expectancy is longer may depend merely on its inherent longevity. It may, however, depend on its relative age at the time when it enters the Gulf. For the latter alternative there is some evidence, for we have found that young individuals of this species appear in the eastern side of the basin during the winter months (Fig. 4). This interpretation renders any special considerations of the relative fitness of the environment for the three species unnecessary, though it does not exclude them as contributory causes.

The distribution within the Gulf appears to depend essentially upon the current system and upon the age of the population which enters the region. This in turn must depend upon the relative remoteness from

the Eastern Channel of the centers of reproduction for each species—a remoteness to be measured in terms of the length and velocity of the transport system which bears the population to its destination.

The Sources of the Deep-water Population

Turning to the external sources of the population which enter the Gulf, the question is: Do the different species have a common source in the slope water which enters the Eastern Channel, or do they come from different sources to be mingled during their passage into the Gulf? The first alternative demands the same relative abundance of the species in the slope water as within the immediate entrance of the Gulf. Huntsman records catches of chaetognaths from 20 stations along the coast of the maritime provinces in water of over 1,000 meters depth made with vertical hauls from depths between 200 or 375 meters and the surface. In these hauls he counted 408 *E. hamata,* 235 *S. maxima,* and 49 *S. lyra.* The numerical ratios are 100: 59: 12. The relative abundance of *S. maxima* and *S. lyra* are almost the same as their occurrence in the Gulf of Maine. *E. hamata* was, however, very much scarcer relatively in these collections than in those made in the Gulf of Maine and was absent from a number of stations at which the others occurred. If Huntsman's catches may be considered characteristic of the slope water to the eastward of the Eastern Channel, then this body—at least in its upper layers—cannot be considered the sole source of the *E. hamata* population of the Gulf of Maine.

Bigelow considered *S. maxima* to come to the Gulf of Maine with the slope water from the northeast. He suggests that *E. hamata* is confined to waters so deep, except in high latitudes, that it never reaches the Gulf of Maine from the oceanic basin abreast of it, a consideration which seems to limit its origin also to the slope water. The absence of *S. lyra* from Huntsman's inshore and eastern stations, together with its more limited distribution in the north Atlantic, seem to preclude its origin from the boreal sources of the slope water. Since Huntsman's and Bigelow's studies were published, Rossby (1936) has shown that there are important forces at work transporting deep oceanic water into the region of the slope water, as the result of which this body owes its origin to oceanic as well as to boreal sources. Redfield (1936) has discussed the ecological consequences of these facts. The diverse origin of the waters which enter the Gulf of Maine and their inhabitants are thus more clearly understood. Hydrographic observations emphasize the complexity of the water bodies occurring close to the mouth of the

Eastern Channel. These facts seem to favor a diversity in the origin of the chaetognaths which penetrate the Eastern Channel.[5]

The Distribution of the Chaetognaths of the Upper Levels

Sagitta enflata, *S. serratodentata*, and *S. elegans*, all of which are more commonly taken in tows from the upper 100 meters, present much greater contrasts in numbers, distribution, and seasonal fluctuations than do the deep-water chaetognaths. Their occurrence and abundance can be attributed to the circulation of water and its relation to the areas suitable for their reproduction in great detail. On the one hand, *S. elegans* is capable of reproducing in the shallow waters which encircle the Gulf and occurs in numbers and at times which can be attributed to the nature and stability of local hydrographic conditions. On the other hand, *S. serratodentata* is an inhabitant of the warm ocean offshore and is carried into the Gulf as a periodic immigrant at times dependent on a cyclic change in the major circulation of the region, while *S. enflata* represents a rare straggler from (hydrographically) more remote regions which can reach the margins of the Gulf only at such times as the temperature of the water permits its survival for a sufficient period.

Sagitta enflata

S. enflata is a tropical form ranging to 40°N in the surface water of the Gulf Stream. Huntsman found it only at the outermost stations along the continental margin and in considerable numbers only at his more western stations in the offing of the Eastern Channel. He captured one specimen in July over the coastal shelf north of Sabro Bank. It has been taken occasionally over the continental slope south of Cape Cod, Bigelow and Sears (1939) recording a large catch of juveniles close to Martha's Vineyard in 1935. South of Delaware it is not infrequent 30 miles in from the continental slope and south of the Chesapeake it occurs frequently close in to land. The invasion of the coastal waters south of Cape Cod occurs at all seasons but most often in the autumn. We captured *S. enflata* on two occasions only. On September 3, 1933

[5] Bigelow found *E. hamata* to vary markedly in abundance from season to season, a fact which might arise from variations in the inflow and its origin from year to year. His capture of *S. maxima* chiefly in winter and *S. lyra* in summer, as well as our failure to find these species dissociated in time may have their origin in similar fluctuations. Bigelow concluded, from hydrographical evidence, that the inflow through the Eastern Channel is variable. Our data have been searched for correlations between the distribution of the species and that of the hydrographic factors in the region of the Eastern Channel, but we have failed to find any good evidence of a dissociation in the time at which the different species enter or in their presence in separate bodies of water within the neighborhood of the Eastern Channel.

one specimen occurred in a haul made in the South Channel in association with large numbers of salpae. On September 23, 1934 five specimens occurred in a haul taken well within the Eastern Channel.

S. enflata is thus one of the many tropical forms which in small numbers find their way in over the margin of the continental shelf in the latter part of each summer. Our records are the first for the margins of the Gulf itself. In the present connection the species is of interest as the extreme example in a series of forms relating frequency of occurrence to the remoteness of the area of reproduction, and to seasonal fluctuations.

S. serratodentata Krohn

This inhabitant of the upper layers of the warmer parts of all oceans extends farther north than most such, being able to survive, though not to breed, in relatively cold water and consequently it extends farther inshore than do most forms of like origin. Huntsman found *S. serratodentata* at practically all stations off the continental slope as far east as the Newfoundland Banks. It extended into the Laurentian Channel over the deep trough but did not penetrate into the Gulf of St. Lawrence. It occurred over the western half of the Nova Scotian Banks well in toward shore and in numbers increasing westward. Bigelow records its occurrence off the continental shelf to the south of Georges Bank and states that from New York southward it is the prevailing chaetognath right in to the shore in warm summers, though outnumbered by *S. elegans* in cooler seasons, at least over the inner part of the shelf as far south as Delaware Bay. North of Chesapeake Bay, and especially east of New York, it is much less frequent close to the land. Bigelow and Sears (1939) find no seasonal fluctuation in its abundance, either inshore or offshore in the coastal belt west of Cape Cod. They consider it to be maintained by local reproduction within that area.

In the Gulf of Maine Bigelow (1926) found it to penetrate the eastern part of the basin regularly and to extend westward along the New England coast in diminishing numbers as far as Massachusetts Bay. He took it only occasionally in small numbers on Georges Bank. It was absent from the southwestern quarter of the basin at all times and disappeared almost completely from the entire Gulf in the late autumn. Its occurrence was highly seasonal, the invasion of the Gulf beginning in late spring and culminating in September. There is no evidence that it reproduces successfully in the Gulf.

Our collections confirm abundantly Bigelow's general conclusions. The center of abundance is in the eastern half of the Gulf, agreeing well with that of the deep-water chaetognaths, though extending into the

shoaler water over Brown's Bank and the Northern Channel. Access to the Gulf is available over this route from the concentrations Huntsman observed on the western Nova Scotia Banks and from the slope water over the Eastern Channel. The inner and western part of the Gulf is penetrated in smaller numbers and in general along the northern side of

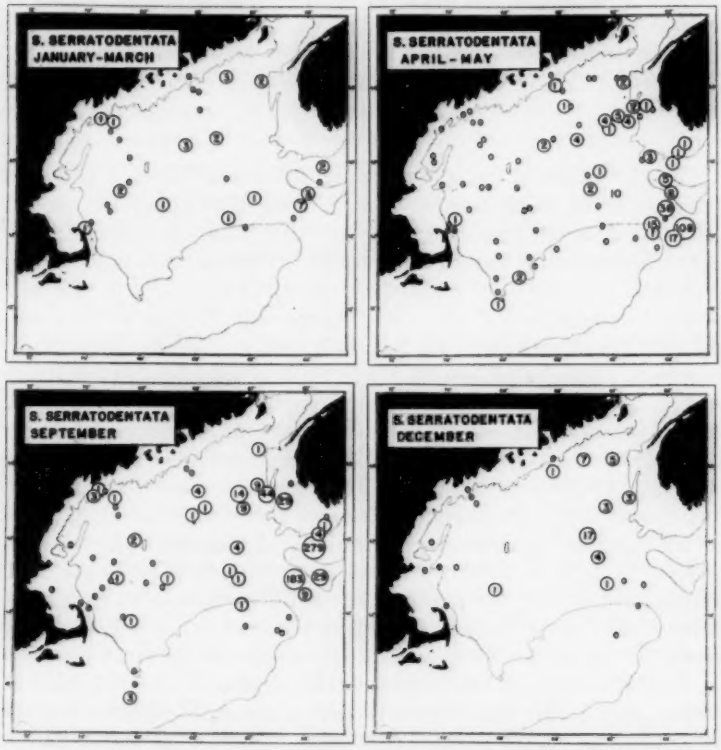

FIG. 9. Locations at which *Sagitta serratodentata* has been taken in the Gulf of Maine at different times of year. Figures in circles represent numbers taken per haul. Small circles represent positions of hauls in which none were taken.

the Gulf. Only in isolated cases were small numbers taken within the 100-meter contour or from the deep basin south and west of Cashes Ledge.

The seasonal picture during 1933–34 is given by Fig. 9, showing the combined catch in cruises grouped according to the time of year. Between January and March the population was at its low ebb, the distribu-

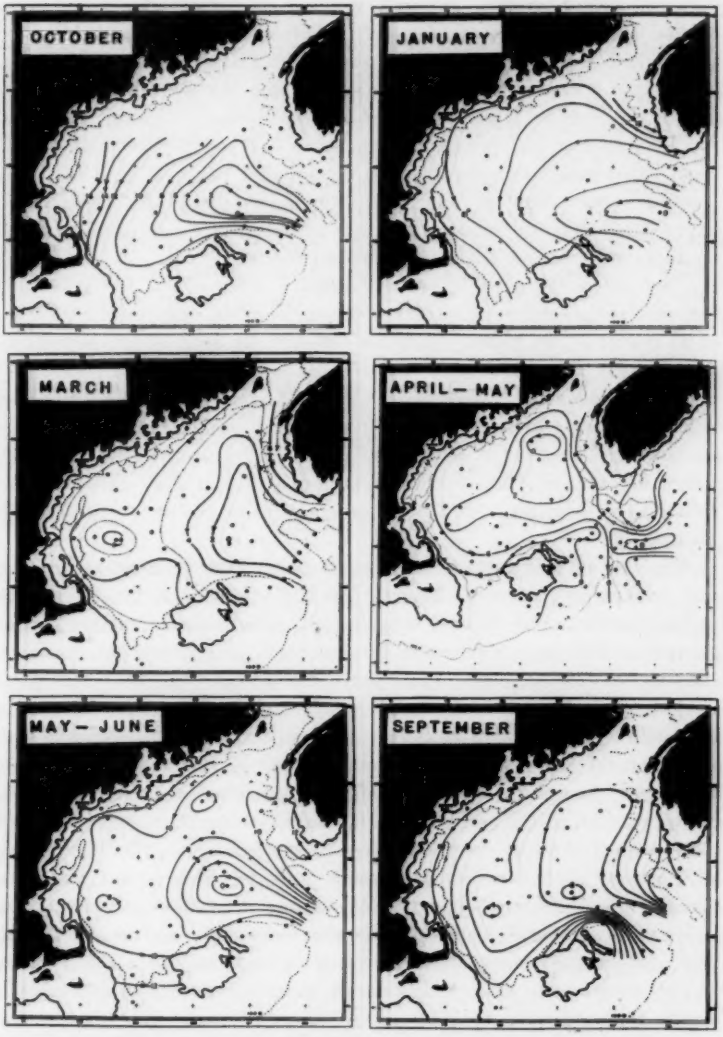

FIG. 10. Dynamic contours showing the theoretical circulation in the Gulf of Maine at a depth of 40 meters from October, 1933 to September, 1934.

tion was almost uniform throughout the Gulf. Such concentration as existed occurred in the northeastern quarter. By April and May the species had disappeared from the western side of the Gulf almost completely, but a new invasion was apparent from the southeast. By September the numbers had increased in this area of concentration and smaller numbers had extended westward as far as Portland while scattered individuals had again reached the remoter parts of the Gulf. By December the inflow of water carrying *S. serratodentata* appeared to have ceased and the remnants of the earlier invasion remain in small numbers still concentrated in the eastern region.

The course taken by *S. serratodentata* in its invasion of the Gulf is that expected of superficial water, entering from the outer Scotian Banks, in its circuit of the Gulf. It follows closely the drift of the population of *Limacina retroversa* which entered the Gulf in December, 1933 and circumnavigated the entire basin (Redfield, 1939). Unlike *Limacina*, this sagitta population did not survive to reach the western basin in significant numbers, and it was rarely found in the southwestern quarter, where lies the most ancient water in the Gulf. It also differed from *Limacina* in invading the Gulf continuously from late April through September.

Bigelow pointed out that neither temperature nor salinity explains the disappearance of *S. serratodentata* in the fall. The water is considerably warmer in November than it is at the time when invasion commences in the spring. Salinity is not very different then from that in late summer. He suggests that increases in numbers in the summer mirror the accumulation of a population which finds conditions favorable there. With the cessation of the invasion in autumn, the visitors of the summer die off from natural causes, leaving no progeny and so the species disappears until new immigrants enter in the spring. This explanation is clearly in accord with the picture we have obtained of the deep-water chaetognaths, save for the periodic character of the invasion.

Bigelow (1926) proposed that the periodic invasion of *S. serratodentata* may be connected with the seasonal reproductive cycle. The lack of any seasonal fluctuations in the numbers taken on the coastal belt west of Cape Cod, subsequently remarked by Bigelow and Sears (1939), leads us to suspect that the periodicity in the Gulf of Maine is due to a periodic change in the circulation of water, rather than to the seasonal character of the reproductive cycle.

The center of reproduction of *S. serratodentata* must lie in the warmer waters east of the continental slope, from which they penetrate the slope water and ultimately reach the Gulf in the indraft over the Eastern Channel. The dynamic contour charts (Fig. 10), showing the

theoretical pattern of circulation in the superficial layers, show that in April–May, 1934 an extensive area of dead water appears to occupy the banks south of Nova Scotia, and that the only apparent movement into the Gulf is from the continental margin off the Eastern Channel. In late May and through October this offshore indraft strengthened, thus supplying the required medium for the invasion of *S. serratodentata* into the Gulf. During the winter months the circulation of sub-surface water into the Gulf alters the contours indicating a much more intense flow across the Scotian Banks. Bigelow (1927) has shown that this change occurs with regularity and results in a regular invasion of water of low temperature and salinity. Such a flow will supply the Gulf with water derived from the inner and more eastern regions of the Scotian Banks where, according to Huntsman (1919), *S. serratodentata* is absent even in summer. During the winter months the invasion of this species into the Gulf will be checked and the population will decline until the inflow of this barren water across the Banks is checked, as it was in April–May, and the offshore indrift becomes predominant.

Sagitta elegans

Sagitta elegans Verrill is the most interesting member of the group because it is the only one maintaining a center of reproduction within the area. It is the characteristic sagitta of the north Atlantic coast, being of general occurrence in the shoaler water. Huntsman found it generally distributed in Canadian waters as far east as the Grand Banks, with particular abundance over the deeper parts of the coastal banks. Offshore it occurs only sparsely beyond the 100-meter contour. Georges Bank is the most southern important center of reproduction, though it ranges south as far as Chesapeake Bay in some seasons (Bigelow and Sears, 1939). In the Gulf of Maine Bigelow found *S. elegans* to occur most plentifully on Georges Bank, in the North Channel and on the adjoining banks south of Cape Sable, in Massachusetts Bay and in smaller numbers along the coast of Maine and New Hampshire within the 100-meter contour. Over the deep basin it is at all times much scarcer. This species becomes very scarce in most parts of the Gulf in early spring. Particularly, in Massachusetts Bay, where dense populations occur in the late summer, it almost entirely disappears in late winter.

Our collections confirm in detail the general features of the distribution of *S. elegans* in the Gulf of Maine described by Bigelow. By combining our data with his records for vertical hauls, and by grouping the two sets of observations by periods in which the findings are essentially

concordant, we are able to give in Fig. 11 a more detailed picture of the seasonal distribution than appeared from his analysis.

Georges Bank was the principal area of abundance of *S. elegans*, both in extent and density of population. Catches of hundreds or thou-

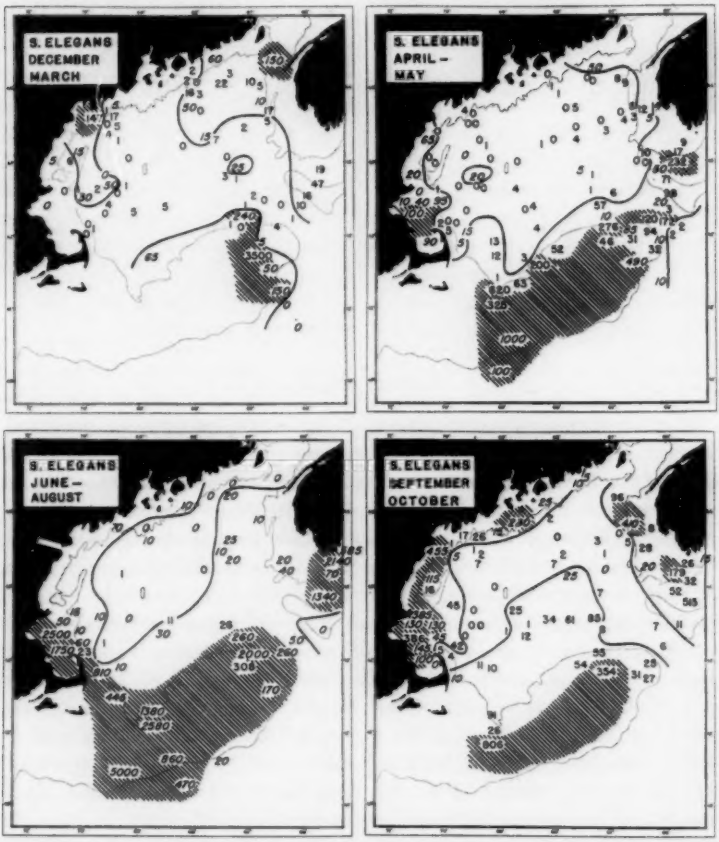

FIG. 11. Locations at which *Sagitta elegans* has been taken in the Gulf of Maine at different times of year. Upright figures represent numbers taken per haul by "Atlantis" in 1933–34. Italic figures represent numbers taken by Bigelow in vertical hauls. Contours enclose areas in which not more than ten specimens per haul were taken.

sands were taken there at all times of year. The area was not adequately explored during the winter months but on the eastern end, at

least, the catches were as abundant then as at any other time. One catch of 3,500 was made in December. The greatest abundance was observed in July.

The Scotian banks south of Cape Sable and the waters over the North Channel was the second area of abundance. In midsummer, catches were obtained there equalling those on Georges Bank. The numbers were less permanent, however, declining in late summer and becoming scanty during the winter period. Recovery was not indicated before May. This area appears to be an extension of the center of abundance which Huntsman (1919) observed on the western Nova Scotia banks in July.

Massachusetts Bay and the coastal waters extending eastward to the Bay of Fundy form an area of intermediate abundance, marked by great seasonal fluctuation. In winter *S. elegans* was rare in these waters with only scattered catches yielding important numbers. In May the population became augmented in Massachusetts and Cape Cod Bays. In midsummer populations rivalling those on the offshore banks developed here and increased numbers were found all along the coast to the eastward by early autumn. These numbers did not maintain themselves, however, with the coming of winter.

The waters over the deep basin of the Gulf and over the Eastern Channel were always scantily populated. There was not much fluctuation in numbers with season in the northern and western parts of the basin. In the southeastern half, particularly in the region north of Georges Bank, there was a distinct increase in the numbers taken, beginning in July and developing clearly in September.

We propose the following considerations in explanation of the observed distribution of *S. elegans* in these different regions. Georges Bank is the seat of a great anticyclonic eddy (Huntsman, 1924; Bigelow, 1927). The dynamic contours in Fig. 6 indicate the magnitude of this eddy. Because of its size and attendant permanence, the population of sagitta remains on the Bank in sufficient numbers to maintain a dense breeding stock. Fluctuations in numbers are only such as are to be expected in an animal reproducing periodically. The decline in numbers at the close of the reproductive season is to be attributed to the natural death of the parental generation and the loss of small numbers to adjoining bodies of water. This situation represents the most favorable condition for maintaining an endemic population. Since, as Russell (1932) has shown, *S. elegans* produces several broods per year, since these are not exactly synchronized, and there is only a short period of

reproductive inactivity in the early winter, fluctuations in numbers are too small to be discerned readily.[6]

The water south of Cape Sable is not recognized as forming a definite eddy, but it is spoken of as a "dead area" subject to strong tidal influence, but for considerable periods relatively free of residual drift. The dynamic contours presented in Fig. 10 show that this condition persisted from April through September in 1934. The freedom from strong currents enables the progeny of this population to accumulate in great numbers during the breeding season. At the end of the period of multiplication the population becomes rapidly scattered, as evidenced by the increased numbers over the coastal banks and deeper waters in the northeastern quarter of the Gulf in the fall and winter.

The alteration of the coastal currents during the winter, discussed in connection with *S. serratodentata,* contributes to the disintegration of this center of abundance. Adjoining extensive breeding areas to the eastward, however, this region remains populated throughout the year. Although the physical and biological conditions south of Cape Sable may be as favorable as on Georges Bank, and the areas to the eastward assure a constant supply of fresh immigrants, the hydrographic set-up does not have sufficient stability to maintain a rich population during the winter.

The coastal waters off the northern shores of the Gulf are the seat of a strong flow to the westward. A non-tidal drift of 7 miles per day is recorded at Portland lightship. Though conditions may not be unsuitable for reproduction in this area, the yield is swept on to the west and south. As a result, only in the relatively immobile waters of Massachusetts Bay is there an opportunity for large numbers to be produced by breeding early in the summer, while the stock grows more slowly in the region to the eastward. With the termination of multiplication the population of the entire coastal zone is rapidly reduced as the waters are swept on, and the population is incorporated into that of Georges Bank and the deeper basin to the north of it.

It is hard to believe that the deeper waters of the Gulf are scantily

[6] *S. elegans* appears to breed in the Gulf of Maine in the late spring and summer. Individuals less than 10 mm. in length may be taken from May to September in Massachusetts Bay and on Georges Bank. Huntsman and Reid (1921) consider the spawning season in the Bay of Fundy to extend from April to September, though the eggs do not develop properly until late summer and the authors doubt if conditions for reproduction are sufficiently favorable in that locality to enable the species to perpetuate itself except by immigration. In the Woods Hole region Fish (1925) found ripe eggs as early as March and April. Russell (1932) has detected four or possibly five successive broods of *S. elegans* in the English Channel, the first appearing as ripe adults in January, the last in October.

populated either because food is lacking or because its physical conditions are unfavorable. Plankton in general is rich in the deep basin, and though perhaps not as plentiful as on the banks, it would surely support more than one-thousandth as many sagitta. The differences in temperature and salinity in the surface waters are trivial. We suggest that the water over the basin is free from *S. elegans* for the same reason that it is populated with *S. serratodentata*. The reciprocal character of the distribution of the two forms is evident both from our own and Huntsman's observations. The superficial water lying over the deep basin of the Gulf is renewed from external sources, in which *S. serratodentata* occurs, but *S. elegans* is lacking. The flow of this water is sufficiently rapid at all times so that *S. elegans* becomes incorporated into it only in small numbers, particularly in the region of inflow and along the northern half of the Gulf. By the time the southern side of the Gulf is reached in the course of the flow about the great eddy which fills the Gulf, *S. serratodentata* has died off, but *S. elegans* has had time to penetrate in larger numbers into the waters overlying the deep basins. This penetration can occur, however, only at those times when a rich population has grown up in the coastal waters, that is, after midsummer, and when this supply is exhausted in early winter the offshore waters become depopulated, both from the death of their inhabitants and their transport out to sea.

Discussion

We have attempted to analyze the distribution and abundance of chaetognaths in the Gulf of Maine on the basis of certain biological characteristics of the species and the movement of the water in which they live. We believe the character of their distribution within the area studied can be accounted for in the main in these terms without recourse to considerations of the suitability of the physical characteristics of the water for survival of the adults or of nutritive conditions.

The biological characteristics taken as given are the depth of water which the species frequents and the character of the water in which it succeeds in breeding. The former is doubtless controlled by choice, tropisms, or avoidance reactions on the part of the individual. Such reactions are left out of account as necessary factors in determining horizontal distribution, it being sufficient to consider that this is determined by the flow and mixing of the water masses in which the animals live. Active swimmers such as the chaetognaths might be capable of definite migrations such as many fish perform. There is no evidence that such directed movements influence the distribution of these forms, though Meek (1928) has claimed that cross-current migrations do influ-

ence the character of the sagitta population along the Northumbrian coast. In the absence of directed migrations, their swimming may be expected merely to accentuate the scattering of the population in horizontal directions at rates greater than the concurrent mixing processes in the water itself.

The situations in which a species can breed are, of course, controlled in some way by the physical characteristics of the water, particularly its temperature. Many species are able to survive as adults in regions in which reproduction is not possible. Five of the six chaetognaths considered fall into this category in the Gulf of Maine. Success in breeding is itself a relative matter and in "marginal" regions insufficient eggs may develop to maintain the local stock. *S. elegans* in the Bay of Fundy appears to be a case in point. There Huntsman and Reid (1921) find that the conditions are unfavorable for the development of larvae and conclude that the very considerable fluctuations in their numbers are caused by the adults being carried into the Bay in varying quantities. We may classify each species as it occurs in a given locality in an order of fecundity which decreases in its favorable effect on the maintenance of the population thus: (1) successful breeder; (2) unsuccessful breeder; (3) non-breeding immigrant.

These categories are merely relative. Success in breeding will affect the abundance of the population both in proportion to the number of eggs spawned and the fraction of these which survive.

Fluctuations in the stock present at any time will depend in part on the purely biological facts relating to the breeding habits. Animals having an annual period of reproduction will show a marked fluctuation with maximal numbers immediately following the period of breeding. The fluctuations will be most marked if the adult does not long survive its first breeding, less pronounced if the adult survives through several breeding seasons. Species which maintain their abundance by the production of many eggs with small expectancy of survival will fluctuate more than those less fecund species with low mortality. The degree to which the reproduction of different species is synchronized with the season will also modify the seasonal incidence. The life histories of the sagittae are not sufficiently well known to permit of classification in these regards. Fluctuations appear to be relatively small in *S. elegans* on Georges Bank, the most stable region of its occurrence. The deep-water species are free from marked fluctuation as is *S. serratodentata* according to Bigelow and Sears (1939) in its reproductive area south of Cape Cod. The marked fluctuations in the latter and the periodic occurrence of *S. enflata* appear to depend on environmental causes, rather than on the biology of the reproductive cycle.

A final biological factor determining both abundance and distribution is longevity. Within a region of production the relation of mortality to reproductive rate is obvious and has been mentioned above in considering fluctuations of biological origin. In the case of immigrant plankton, which are carried beyond the areas of successful breeding, the longevity of the individuals will determine the distance which may be travelled and the relative numbers to be found in regions increasingly remote from the centers of production. The deep-water chaetognaths afford examples of this sort as do *S. serratodentata* and *S. lyra*.

In nature these biological factors are at play in a scene determined by the given hydrography of various regions. The permanence of a maximal population demands that the breeding stock shall not be scattered by the flow of water, or that such losses shall be balanced by recruitment from other rich areas. The most favorable conditions for endemic existence will be found in large areas where suitable conditions are found and in which the water remains more or less permanently. Such an area is provided by the great eddy which appears to exist on Georges Bank and here *S. elegans* maintains a dense and stable population in spite of the fact that there is little opportunity of recruitment from other regions. Russell (1936*b*) considers the source of the water which occasionally carries *S. elegans* into the English Channel to be an area of cyclonic circulation lying south of Ireland.

When opportunities for recruitment by immigration from adjoining areas are good, rich populations may be developed in regions which do not develop permanent eddies in spite of a relatively rapid dissipation, as in the case of the banks south of Cape Sable. In such areas the seasonal fluctuations become greater and increasingly so in small areas such as Massachusetts Bay in which little time is required to wash away the dense populations, as soon as the period of multiplication is passed. In such regions the population is pseudo-endemic inasmuch as it is dependent on immigration to a high degree.

Bodies of water originating in regions where a species cannot maintain a production center may acquire a population by immigration from such centers in the course of its drift. The scanty populations of *S. elegans* which are found in the waters over the central basin of the Gulf of Maine appear to be of this type. Such populations become richer the longer the water is in proximity to the source area of production. Whether the species can reproduce in the area or not, it cannot achieve significant numbers because the water moves on too rapidly to permit an accumulation. Such populations may show seasonal fluctuations reflecting the reproduction periods of the areas from which they are derived, as in the southern half of the basin.

The origin of water bodies having distinctive physical characteristics results primarily from the mixing of other characteristic waters. The formation of slope water through the admixture of tropical, boreal and coastal waters is a case in point. In its synthesis the inhabitants of the contributing regions become incorporated into it and are carried with it to regions remote from their centers of reproduction. Such immigrants, if unable to reproduce themselves, can travel only so far as their life span will permit. They may be regarded as terminal immigrants in contrast to the forms such as *S. elegans* in Massachusetts Bay which form pseudo-endemic populations in regions continuously swept out by the passing waters. *E. hamata, S. maxima* and *S. lyra* are examples of this category.

Immigrants, both terminal and pseudoendemic, may show fluctuations both as the result of the reproductive cycle and for hydrographic causes. While the fluctuation of the pseudo-endemic population of *S. elegans* in Massachusetts Bay is probably due to seasonal reproduction in an area of relatively constant dissipation by currents, a changing current pattern may contribute largely to the fluctuation of the population of this species off Cape Sable. *Limacina retroversa* fluctuates greatly in numbers in the Gulf because it enters intermittently presumably because of fluctuations in the sources of the water entering the Gulf. Though it may be classed as an unsuccessful breeder in the Gulf, the important reason for its failure to develop a truly endemic population is the rapid flow of water across the surface of the basin which carries it away following its intermittent periods of invasion. *Limacina retroversa* is thus an unsuccessful pseudo-endemic form fluctuating for hydrographic reasons.

S. serratodentata, essentially a terminal immigrant in the Gulf of Maine, fluctuates in its occurrence for reasons which appear to be largely hydrographic. Being unable to survive long after the cessation of the inflow which introduces it into the Gulf, its fluctuations so far as this area is concerned are more marked than any others. *S. enflata,* occurring as a rare straggler along the borders of the Gulf, is the extreme case of terminal immigration. Its occurrence in late summer, along with many other tropical forms, suggests a definite movement of tropical water onto our shores at that time. The water in which these forms occur does not differ from normal coastal water in salinity. Their presence is due to the admixture of exceedingly small amounts of tropical water into that of the coastal banks. Their seasonal occurrence is to be related to the annual temperature cycle of the coastal waters. Unable to survive the chilling of winter, these rare stragglers are unable to travel far from their production areas beyond the slope water until the latter part of summer.

Among the chaetognaths, only one—*S. elegans*—has established a truly endemic existence in the Gulf of Maine, and this on the coastal and offshore banks. It alone achieves important numbers for this reason, and it alone is sufficiently numerous so that nutritive conditions may limit its numbers. The other species are immigrants from other regions and occur in numbers depending on the remoteness of their areas of production and no doubt on their abundance in such regions.

We may inquire why the basin of the Gulf has acquired no successful endemic population of chaetognaths. So far as the surface waters are concerned, the answer seems to be that these circulate so rapidly that time is not permitted for the accumulation of even a considerable pseudo-endemic population. The evidence is found in the history of *Limacina retroversa* and of *S. elegans* in the southern half of the basin. In the deeper water this difficulty can scarcely obtain. These waters derived from the slope water undergo rapid modification within the Gulf by admixture with surface water and acquire lower temperatures and salinities than those characterizing the slope water at comparable depths, and lower salinities than slope water of the same temperature. The process of creating a characteristic water mass, such as the slope water is, is continuing actively in the basin of the Gulf. Perhaps for this reason no chaetognath has yet appeared capable of reproducing successfully in this unique and limited region. However this may be, it is clear that the basin of the Gulf does support a rich endemic population of Crustacea. How this is possible should be the subject of future study.

Summary

1. Data are presented concerning the distribution and numerical abundance of five species of chaetognaths taken in the Gulf of Maine during the year 1933–34 and the hydrographic features controlling their abundance is discussed.

2. It is concluded that *Eukrohnia hamata, Sagitta maxima* and *S. lyra,* which are carried into the Gulf by deep currents and do not breed there, occur in numbers which depend not only on their relative abundance in various offshore waters which mingle in the Gulf, but on their longevity after entering the Gulf.

3. *Sagitta serratodentata* is a terminal immigrant from the superficial waters of the Atlantic which fluctuates in its abundance as the result of periodic changes in the circulation of water entering the Gulf from the east.

4. *Sagitta elegans* is the only chaetognath truly endemic to the region. The permanence of its occurrence appears to depend on the presence of

a relatively stable eddy on Georges Bank. Its occurrence in other regions varies with the season to a degree which may be explained by local conditions of circulation.

5. *Sagitta enflata* is recorded for the first time from the margins of the Gulf of Maine.

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STUDIES ON THE PROTEINS OF SMOOTH MUSCLE

II. The Myosins of the Octopus, Snail, Sea Cucumber and Sea Anemone

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Despite great advances in our knowledge of the composition of muscle, and of the chemical reactions which accompany contraction, it cannot yet be said that we have any real understanding of the process of muscular contraction. One of the obstacles in the way of such an understanding is our lack of knowledge regarding the position of the muscle proteins, and their rôle in contraction. From the standpoint of structure, myosin appears to be the most important protein in muscle. The available evidence indicates that myosin accounts for from 40 to 50 per cent of the total proteins of muscle (1) (2) (3) (4). Both the optical properties and the X-ray diffraction patterns of muscle fibres and myosin fibres show a striking correspondence (5) (6) (7) (8) (9). It would therefore appear reasonable to suppose that the muscle fibril is essentially a fibre of oriented myosin molecules, and that contraction is associated with a contraction of this fibre. There are, however, a number of unsolved problems, such as the significance of the anistropic and isotropic bands (striations) in striated muscle and the existence or non-existence of certain membranes across the fibre axis. Some aspects of these problems have been discussed by Weber (3) (4) and by Bernal (10).

Because of its apparently greater structural simplicity, smooth muscle might seem to be a better system for investigation. Under any circumstances, any general theory of the place of myosin or other proteins in muscular contraction must consider the properties of both smooth and striated muscle. In particular, it seems desirable to extend our information regarding the properties and distribution of myosin. Previous studies have been limited largely to the striated muscle of the vertebrates, although von Muralt and Edsall (11) have reported the isolation of myosin from the smooth muscle of the marine snail, *Busycon*. More recently myosin has been prepared from lobster muscle (12). Bailey also studied the composition of the myosins of skeletal muscle of several

[1] These studies were made at Kerckhoff Marine Laboratory of the California Institute of Technology, Corona Del Mar, California.

species of vertebrates. In addition to the study of composition, it would be desirable to investigate the variation of the physical properties of myosin in different animals and in different types of muscle.

Two very characteristic properties, which serve to distinguish myosin from other muscle proteins, are its solubility and double refraction of flow. The solubility properties of myosin make the preparation a relatively simple process; while the double refraction of flow, which is indicative of the rod, or thread-like shape of the myosin molecule, may be used to characterize the preparations obtained. With the construction of a portable apparatus for the study of double refraction of flow, a brief, exploratory excursion into marine biology seemed attractive. The results obtained, though limited, indicate certain interesting possibilities and problems.

Methods

The preparation of myosin is based upon its characteristic solubility properties (1) (2) (3) (13) (14) (15). Myosin is insoluble in salt solutions and water at pH 5 to 6, but soluble in moderately concentrated salt solutions on the alkaline side of the isoelectric point. It is, however, relatively insoluble in dilute salt solutions at pH 6 to 7. In the method used, the tissue is thoroughly minced and extracted with 0.5 to 1 M KCl which contains 0.02 M $NaHCO_3$ or phosphate buffer with a pH of 7 or greater. The extract is filtered through cheesecloth and then through paper pulp. The filtrate is then adjusted to about pH 6.5 and diluted with 5 to 10 volumes of water. This should cause the myosin to precipitate in a flocculent form which will settle out slowly. After this preliminary concentration of the precipitate by settling, it can be further concentrated by centrifugation. The precipitate is washed with water, and is finally dissolved by adding sufficient saturated KCl solution to give a solution of 0.5 M to 1 M. It is desirable to carry out all the procedures in the cold, but in the present experiments only the extraction and the preliminary concentration of the precipitated myosin by settling could be done in the cold. Recent studies (15) (16) have shown that the behavior of myosin preparations, particularly with respect to double refraction of flow, may be markedly influenced by a variety of factors. Most salts have a considerable effect, and even KCl in too high concentrations may result in reduced double refraction of flow. For that reason, the data presented cannot be assumed to represent values for completely unaltered myosin. A more detailed study of each myosin will be needed to determine the particularly important factors in each case.

The apparatus used for the study of double refraction of flow is, in

principle, the same as that previously used in this laboratory (11) (17) (25).[2] Solutions which show double refraction of flow,—that is, become optically anisotropic when subjected to shearing forces,—may be best studied by placing them between two concentric cylinders, one of which rotates, while the other is held fixed. The shearing forces resulting from the velocity gradient produced in the liquid in this way will be uniform along any line through the liquid, parallel to the axis of the cylinders. The optical properties will therefore be uniform along such a line, and may be conveniently observed with a suitable optical system. The principles of these measurements are discussed in the papers referred to above (11) (17) (25), as well as in a number of other papers (19) (20) (24).

Two variables are studied as a function of the velocity gradient, the extinction angle, and the amount of double refraction. The extinction angle is the angle between the optical axis in the bi-refringent solution and the tangent to the circumference of the concentric cylinders at the radius on which the point of observation lies. According to the theory of double refraction of flow (20) (21) (22), the direction of the optical axis will generally correspond to the principal direction of orientation of the molecules, so the extinction angle will give the angle between the principal direction of orientation of the long axes of the molecules and the tangent to the circumference of the cylinders. When the velocity gradient approaches zero, the orientation becomes more nearly random, and the extinction angle approaches 45°. At higher velocity gradients the molecules become more highly oriented, and tend to lie with their long axes parallel to the tangent to the circumference. Thus, at high velocity gradients, the extinction angle will approach 0° as a limiting value. The ease with which the molecules may be oriented depends upon their size and shape, and an approximate relation has been obtained for the extinction angle, χ, as a function of the ratio of the velocity gradient, β, to the rotatory diffusion constant, Θ (20) (21). With reference to the results to be presented, it may simply be said that the longer the molecule, the smaller the extinction angle will be at a given velocity gradient. Since the rotatory diffusion constant is roughly inversely proportional to the cube of the length of the molecule, the extinction angle will be quite sensitive to the length of the molecule.

The amount of double refraction is given by the difference between the refractive indices of the solution for the extraordinary and for the ordinary ray, $n_e - n_o$. The value of $n_e - n_o$ is zero for zero velocity gradient, and increases toward some upper limit as the velocity gradient

[2] A complete description of the new apparatus will be given in a future publication.

is increased. The upper limit corresponds to the value of $n_e - n_o$ for complete orientation of all the molecules. Practically, it is not possible to reach this upper limit, nor can it be readily calculated; but it will depend upon the specific optical properties of the molecules,—upon their refractive index and that solvent, and upon any double refraction inherent in the molecule itself. The amount of double refraction will also depend upon the concentration of the solution, and in dilute solution will be proportional to the concentration.

It will be seen that the extinction angle is a function, at least in dilute solution, of the velocity gradient and the rotatory diffusion constant alone. It is, therefore, the most useful value for estimating the size and shape of the molecule under consideration. The amount of double refraction is a function of the optical properties as well, and cannot be used alone to obtain information regarding either size and shape, or the optical properties of the molecule. When, however, different solutions are compared at equal values of the extinction angle (that is, at equal degrees of orientation), a comparison of the amounts of double refraction does give some indication of the inherent optical properties of the molecules. Measurements of $n_e - n_o$ are also useful for measurements of the rate of loss of double refraction of flow under various conditions.

The nitrogen determinations were made by the micro-Kjeldahl method. pH determinations were, for the most part, very roughly made, using indicators. In several cases, however, these values were checked by measurements with the glass electrode.

Materials

The Sting Ray (*Urobatis halleri*)

The tail and wing muscles [3] of one ray were ground and extracted in the cold with 1 M KCl + 0.02 M $NaHCO_3$. After precipitation of the myosin it was dissolved by adding KCl to make the solution 1 M. The solution was very turbid and was not readily clarified, so only a limited number of experiments were made with it.

The Octopus (*Polypus bimaculatus*)

The first preparation was made by extracting the ground tentacles with 1 M KCl + 0.02 M $NaHCO_3$. Considerable difficulty was experienced with turbidity in the filtrates, and when this myosin was finally redissolved in 1 M KCl it showed no double refraction of flow. A second preparation was then made, this time using 0.5 M KCl + 0.02 N $NaHCO_3$ for the extraction. This gave a solution of myosin which,

[3] These muscles are striated.

though turbid, showed strong double refraction of flow. Precipitation upon dilution occurred at a pH as high as 7, although the point of maximum flocculation upon the addition of acid was around pH 4.5 to 5.5.

The Snail (Polynices lewisii)

The foot muscle from two snails was ground and extracted with 0.5 M KCl + 0.02 M $NaHCO_3$. In adjusting the pH for precipitation there was a very marked tendency for precipitate to form at the addition of each drop of acid, and a precipitate could be obtained upon dilution at a pH as high as 8. The solution of myosin finally obtained was quite turbid, but showed marked double refraction of flow.

The Sea Anemone (Cribrina xantho-grammica)

The whole animal was ground and extracted with 0.5 M KCl + 0.02 M $NaHCO_3$. The filtrate showed no double refraction of flow, and acidification with HOAc gave only a small amount of precipitate. The precipitate would not redissolve in KCl, and seemed to be due to the aggregation of suspended material.

The Sea Cucumber (Stichopus californicus)

The cutis and muscularis were treated separately. Each was extracted with 0.5 M KCl + 0.02 M PO_4 at pH 7.5. Neither extract showed double refraction of flow, any precipitate on dilution at pH 6.5, or more than a small amount of precipitation at pH 5. A second extract was made, this time using phosphate buffer with a pH of 8.5, but these extracts behaved like the first pair.

Results

The measurements of the extinction position in the solution are shown in Fig. 1. The extinction angle is given as a function of the velocity gradient within the solution. All of these measurements were made in concentrations at which the position of the optical axis is affected somewhat by concentration, so solutions of about the same concentration have been chosen for comparison. The measurements on the octopus and snail myosins were made at about 25° C., and are not strictly comparable with the values for rabbit myosin,[4] which were made at 3° (17). The values for Ray myosin,[5] which are not shown, would indicate that it is similar to octopus myosin.

[4] The muscles from which the myosins of the sting ray and rabbit were prepared are striated.

[5] The isolation of myosin from mammalian intestinal muscle has been reported (27). Certain atypical properties make it seem desirable to withhold final judgment on that case pending further investigation.

Figure 2 gives the values of the amount of double refraction of flow as a function of the velocity gradient. Because of the approximate proportionality between the amount of double refraction at a given velocity gradient and the concentration, the values of $n_e - n_o$ have been divided by the concentration of the protein in grams per cc. of solution, using a nitrogen factor of 6.45. The snail myosin gives values which are higher than those for the octopus myosin at equal velocity gradients. This is due, however, to the greater degree of orientation of the snail myosin. If the values of the double refraction are compared at equal values of the extinction angle, the double refraction of the octopus myosin would

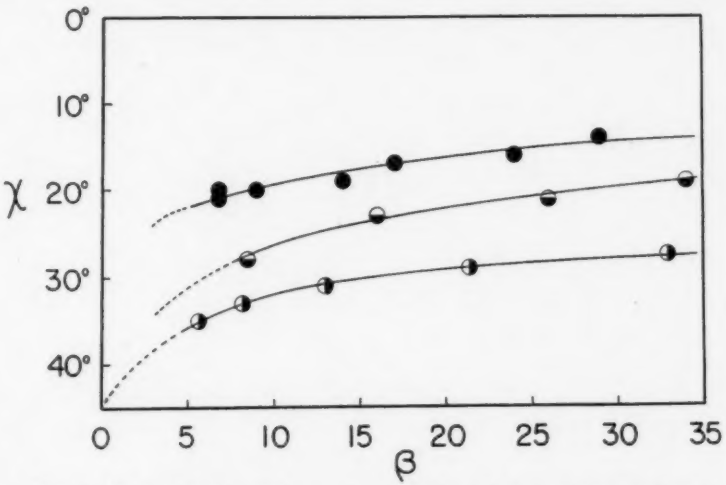

FIG. 1. The extinction angle, χ, as a function of the velocity gradient, β. ●—snail myosin at 25°, nitrogen = 5.3×10^{-4} gram/cc.; ◑—octopus myosin at 25°, nitrogen = 5.9×10^{-4} gram/cc., 25°; ◒—rabbit myosin at 3°, 4.5×10^{-4} gram/cc., from the measurements of von Muralt and Edsall (17).

seem to be greater. Moreover, the value of the double refraction of the snail myosin is about equal to, or slightly greater than, that of the rabbit myosin at equal values of the extinction position. Here again the measurements on the rabbit myosin were made at 3° (11), while the measurements of this investigation were made at 25°. The effect of temperature upon the amount of double refraction is quite marked (11), and the change from 3° to 25° would reduce the double refraction by about 50 per cent. Cow myosin gives values of the angle of isocline comparable with rabbit myosin, but has about twice the amount of double refraction (11) (17).

The effect of alkali on these myosins is similar to the effects already reported for cow and rabbit myosin (11). Measurements of the amount of double refraction were made at constant velocity gradient, as a function of time. The double refraction of flow decreases rapidly at pH 10. A decrease is obtained with octopus myosin at pH 8, the loss being about 50 per cent in 45 minutes. At pH 9 a decrease to 50 per cent of the initial value takes place in about 25 minutes. Essentially the same result was obtained with snail myosin.

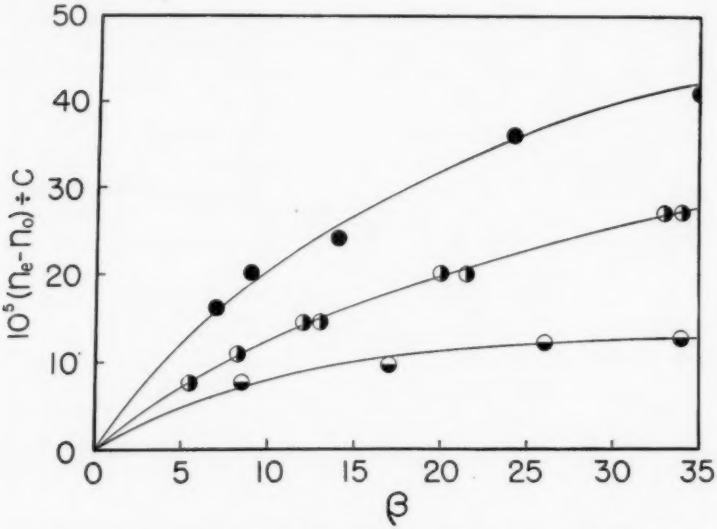

FIG. 2. The amount of double refraction $(n_e - n_o) \div C$, as a function of the velocity gradient, β. ●—snail myosin at 25°, nitrogen = 5.3×10^{-4} gram/cc.; ◑—octopus myosin at 25°, nitrogen = 5.9×10^{-4} gram/cc.; ◒—rabbit myosin at 3°, nitrogen = 4.5×10^{-4} gram/cc., from the measurements of von Muralt and Edsall (11).

The effect of urea upon the double refraction of flow was also studied. The results obtained are shown in Fig. 3, where the percentage of the original amount of double refraction is given as a function of the time. There is a general similarity between the different myosins, with secondary differences. It is perhaps of some interest that the ray myosin is no less sensitive to the effects of urea than are the other myosins, although the uremia of the elasmobranchs (about 2 per cent urea in marine elasmobranchs) is of the same order of magnitude as the concentrations of urea needed to produce denaturation (about 3–4 per cent).

Discussion

Perhaps the most notable feature of these results is that the differences between the mollusk myosins and the vertebrate myosins are no

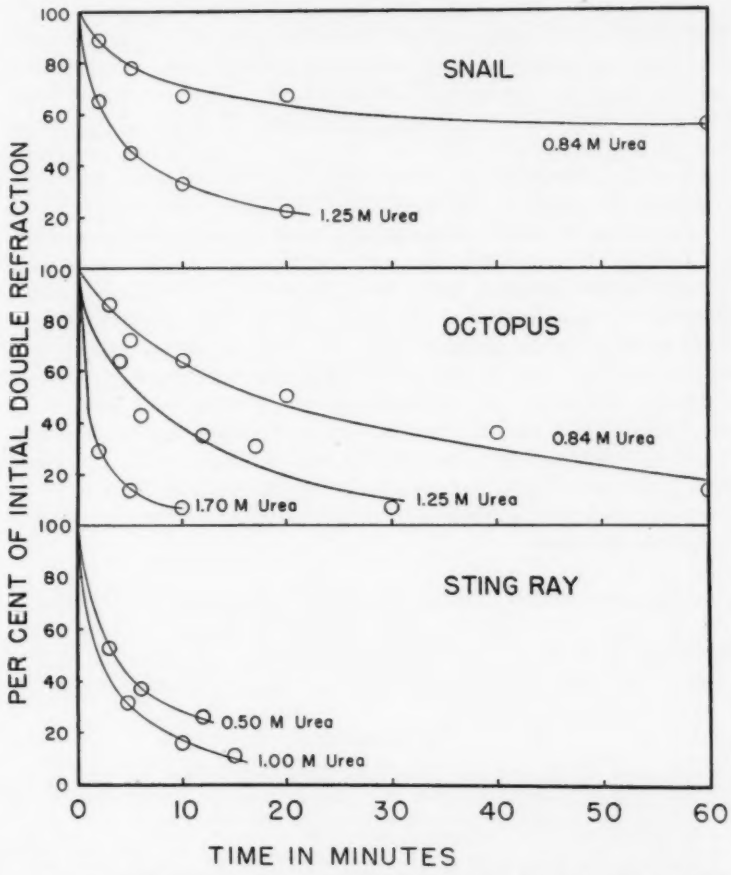

Fig. 3. The effect of urea upon the amount of double refraction of flow. Percentage of original double refraction of flow as a function of time after mixing with urea to give the final concentration of urea indicated.

greater than the differences between the myosins from the two species of mollusks, or between the different vertebrate myosins. From measurements of double refraction of flow, it has been estimated that the length

of the rabbit myosin molecule is between 5,000 and 10,000 A (25). Calculations made in the same way would place the length of the octopus myosin at 5,000 to 10,000 A, the snail myosin at 13,000 to 27,000 A, and the ray myosin at 5,000 to 10,000 A. This is in keeping with the general similarity in the amino acid composition of the various myosins. This similarity in composition, the characteristic solubility, and the common large size and great asymmetry indicated by double refraction of flow, all point to a common architectural plan. The fact that the same protein is found in both smooth and striated muscle would indicate that, at least in certain cases,[5] the two types of muscle differ with respect to the arrangement of the proteins within them rather than with respect to the nature of the proteins of which they are composed.

The failure to isolate such a protein from the sea cucumber and the anemone would be as interesting and significant if it were not possible that the negative result is due to the protein being less accessible to extraction—perhaps because disintegration of the cells is more difficult. In the case of the sea cucumber cutis, the X-ray results of Astbury and co-workers (28) make it seem likely that the doubly refracting fibres are composed of a protein of the collagen group. Further work is necessary to elucidate the position of the sea cucumber muscle, and to determine whether myosin will be found in other invertebrate muscles. It would seem to be particularly important to investigate the possibility of a correlation between the properties of muscle proteins and the physiological properties of muscle.

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NOTES ON THE PHARMACOLOGY OF THE HEARTS OF ARIOLIMAX COLUMBIANUS AND ASTACUS TROWBRIDGEI

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In the Mollusca the mediator acetylcholine has been found to be a cardiac inhibitor, while in the Arthropoda it is strongly stimulatory to cardiac action. Atropine has been found to abolish the action of acetylcholine in Arthropoda (Welsh, 1939*a*, 1939*b*; Hamilton, 1939) but not in Mollusca (Jullien, 1936; Prosser, 1940, etc.). In both groups, as well as in many other invertebrates and vertebrates, it has been found that eserine sensitizes muscle tissue to acetylcholine by rendering inactive the cholinesterase present in the tissue, and perhaps because there exists a synergism between acetylcholine and eserine (Freud and Uyldert, 1936). Nicotine is known to act on ganglionic cells, thus blocking nerve impulses. In the vertebrates in small doses it leads to temporary stimulation followed by depression, and in large doses depression is evident immediately.

ARIOLIMAX COLUMBIANUS

The investigation of molluscan hearts to determine whether or not a cholinergic system of heart control is present has occupied the interest of physiologists for several years. The mollusks are a diverse group; this fact is emphasized by consideration of a brief tabulation of findings regarding the cholinergic physiology of the hearts of various forms (Table I). It is interesting that Prosser (1940) finds that the sensitivity of the heart of the clam, *Venus*, to acetylcholine varies greatly from spring to fall. Included in the table are the experimental results for the slug *Ariolimax*.

In the slug the heart, kidney and lung make up the pallial complex which almost fills the mantle cavity. The heart, which lies on the dorsal surface of the kidney, consists of a very thin-walled auricle and a ventricle from which issues a large aorta; it is essentially a muscular tube constricted at one point.

The animal is fastened to wax with pins through the foot at 2 or 3 cm. intervals. A small incision is made under the posterior flap of the

mantle about 1 cm. to the left of the median line; the cut is carried forward to the anterior edge of the mantle. The skin on the right side of the incision is lifted and cut away as necessary, disclosing the delicate pericardium, under which can be seen the beating heart. In cutting the pericardium, great care must be taken not to damage the ventricle, which responds to harsh, direct contact or injury by going into systolic tetany.

The aorta is cut about 5 mm. from its origin, and the heart is freed from pericardial and renal tissue; the auricle is severed about 3 mm. from the auriculo-ventricular junction, and the ventricle is transferred immediately to the physiological solution. The aortal stub is secured to a small steel hook fixed in a bent glass rod, and a small glass hook suspended by a thread from the recording lever is passed through a hole made through the remaining auricular tissue. When tension is applied, the ventricle beats rhythmically, shortening along the vertical axis.

The isolated ventricle is completely bathed in a desired solution by raising a beaker containing that solution around the support of the fixed steel hook. By using this method it is possible to change the solution quickly and smoothly without disturbing the record.

The most favorable salt solution used was Cardot's (1921) *Helix* Ringer. Salts were dissolved in distilled water in the following concentrations: (grams per liter) NaCl 9.0; KCl 0.42; $CaCl_2$ 0.24. Possibly this Ringer did not have a perfect ionic balance nor complete isotonicity to the plasma of *Ariolimax*; the ventricle remained active for only a few hours after excision.

The temperature effects mentioned by Carlson (1906) were found to hold true in these experiments. At about 16° C. the normal heart rate consisted of about 35 ventricular systoles per minute; at 22° C. there were 57 beats per minute. Thus, for every degree rise in temperature, there was an increase of about 3.6 beats per minute. Recording was done in a cold-room with temperature of 14–18° C. The temperature remained constant during a given experiment; uniform rate of beat was thus insured.

Acetylcholine chloride Merck was kept on ice in stock solutions of .1 gram in 100 cc. of .01 N HCl. Aqueous stock solutions of 1:1000 eserine salicylate Merck, atropine sulphate Merck, and nicotine Eastman were also kept on ice, and with acetylcholine were freshly prepared every 2 or 3 days.

Acetylcholine

Acetylcholine has negative inotropic and negative chronotropic effects on the ventricle of *Ariolimax*. The first definite indication of an effect is seen at 1:10 million (Fig. 1*B*), and this concentration may be

TABLE I

The effect of drugs on molluscan hearts.

Animal	Acetylcholine Effect	Acetylcholine Effect after Eserine	Atropine Effect	Acetylcholine Effect after Atropine
Oyster (Jullien, 1936)	Threshold 1 : 100,000. Diastolic stoppage between 1 : 50,000 and 1 : 5,000.	***	1 : 1000 gives pos. ino- and neg. chronotropic effects.	No antagonism
Clam *Venus mercenaria* (Prosser, 1940)	Threshold 1 : 1 trillion in spring; 1 : 10 billion in fall.	Increased sensitivity to acetylcholine. Prolonged inhibition by acetylcholine.	1 : 10,000 toxic	No antagonism
Snail *Helix sp.* (Jullien, 1936)	Threshold 1 : 1 million. Diastolic stoppage at 1 : 50,000	***	1 : 20,000 gives pos. chrono- and small neg. inotropic effects.	No antagonism
Snail *Murex sp.* (Jullien, 1936)	Threshold 1 : 1 million. Diastolic stoppage at 1 : 50,000	***	1 : 2500 starts heart which has not started to beat.	No antagonism
Slug *Ariolimax columbianus*	Threshold 1 : 10 million. Diastolic stoppage at 1 : 50,000.	Increased sensitivity to acetylcholine	1 : 1000 toxic	No antagonism
Squid *Loligo pealii* (Bacq, 1934)	Threshold 1 : 1 billion. Diastolic stoppage at 1 : 10 million.	***	1 : 1 million gives negative inotropic and chronotropic effects.	No antagonism

considered the threshold. At progressively stronger concentrations the effects become more pronounced until at a concentration of 1 : 50,000 diastolic stoppage occurs (Fig. 1*C*). Normal beat is always resumed upon replacement of the acetylcholine solution by Ringer. After diastolic stoppage of a heart by acetylcholine, direct local electrical stimulation causes a strong contraction.

Eserine

A heart was bathed for 20 minutes in a 1 : 1 million solution of eserine, which has only a slight slowing effect; during this time the solution was frequently washed. In this eserinized heart the acetylcholine threshold was lowered to 1 : 100 million (Fig. 1*D*). Diastolic stoppage oc-

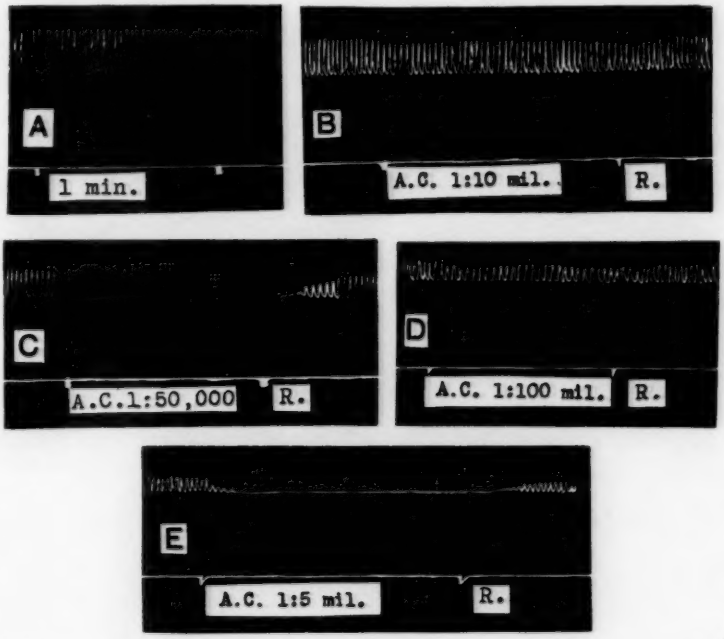

FIG. 1. *Ariolimax*. *A*, normal beat at 16° C. *B*, effect of acetylcholine 1 : 10,000,000. *C*, effect of acetylcholine 1 : 50,000. *D*, effect of acetylcholine 1 : 100,000,000 on ventricle treated for 20 minutes with eserine 1 : 1,000,000. *E*, effect of acetylcholine 1 : 5,000,000 on ventricle treated for 20 minutes with eserine 1 : 1,000,000.

curred with acetylcholine 1 : 5 million (Fig. 1*E*), that is, with a solution of acetylcholine 100 times more dilute than is necessary for stoppage of an un-eserinized heart.

Atropine

Atropine has no effect except in toxic concentration (1 : 1000). After administration of a non-toxic solution of atropine (1 : 10,000) or in

mixture with such a solution (Fig. 2*A*), acetylcholine 1 : 50,000 carries out its characteristic stoppage effect without alteration.

Nicotine

Positive inotropic and negative chronotropic effects were produced by this drug in low concentrations. The first noticeable effect was a slowing of the rhythm by nicotine 1 : 7000. With nicotine 1 :5000 a definite negative chronotropic effect could be observed, and to this effect

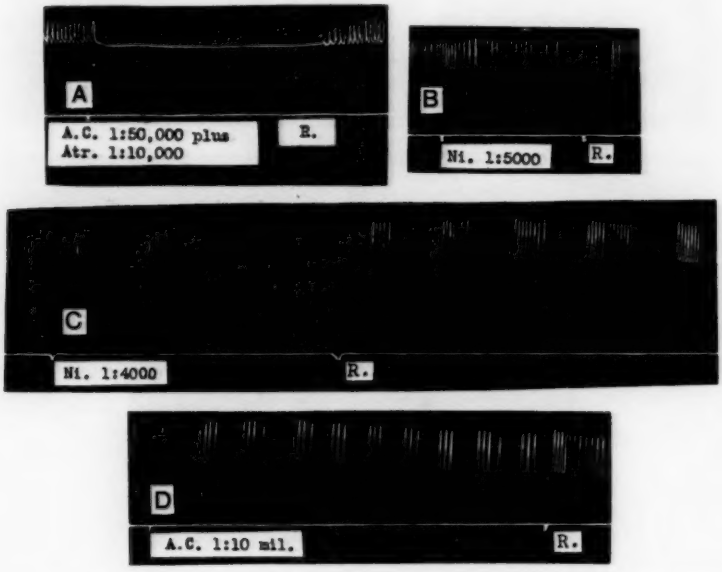

FIG. 2. *Ariolimax*. *A*, effect of acetylcholine 1 : 50,000 in mixture with atropine 1 : 10,000. *B*, effect of nicotine 1 : 5000. *C*, effect of nicotine 1 :4000. *D*, effect of acetylcholine 1 : 10,000,000 after treatment for 13 minutes with nicotine 1 : 5000.

was added an increase in magnitude of contraction (Fig. 2*B*). With nicotine 1 : 4000 diastolic stoppage with a few irregular beats of large magnitude occurred (Fig. 2*C*). The recovery from nicotine 1 : 4000 was interesting. After the nicotine solution was replaced by fresh Ringer, the heart beat five times with large amplitude and then stopped in diastole for a short time. This stoppage was followed by several strong beats, and that process of periodic stoppage, followed in each case

by a series of beats of increasing number, continued until the heart had resumed a normal though amplified rhythm. Nicotine 1 : 3000 caused complete diastolic stoppage, with recovery similar to that from nicotine 1 : 4000. Nicotine 1 : 1000 was toxic.

A heart was bathed for 13 minutes in 1 : 5000 solution of nicotine and maintained a slower though amplified beat; acetylcholine 1 : 10 million produced a periodic stoppage and resumption of beat (Fig. 2*D*). Acetylcholine 1 : 100,000 caused diastolic stoppage of a heart nicotinized with a 1 : 5000 solution.

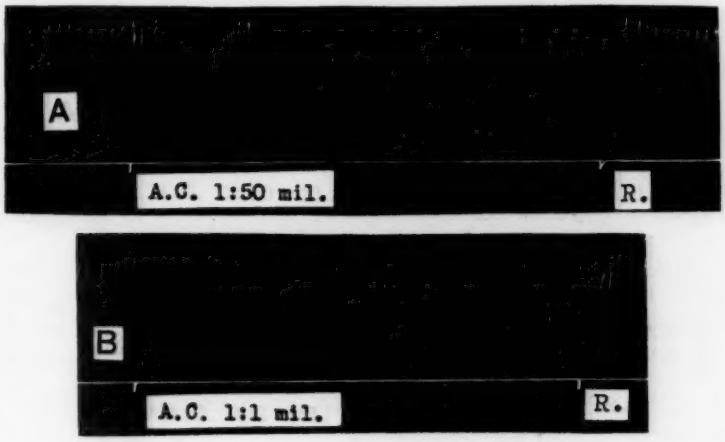

FIG. 3. *Ariolimax*. A, effect of acetylcholine 1 : 50,000,000 after treatment for 27 minutes with nicotine 1 : 8000. *B*, effect of acetylcholine 1 : 1,000,000 after treatment for 27 minutes with nicotine 1 : 8000.

After nicotinization of a heart with a 1 : 8000 nicotine solution for 27 minutes, acetylcholine effects were somewhat more characteristic. The most dilute acetylcholine solution to produce a noticeable effect was 1 : 500 million. This effect became more pronounced with progressively stronger solutions (Fig. 3*A*) until at acetylcholine 1 : 1 million diastolic stoppage occurred (Fig. 3*B*).

After treatment with nicotine 1 : 3000, which causes diastolic stoppage, a heart responded to local electrical stimulation with a strong contraction.

Conclusions

Acetylcholine apparently takes effect on the pacemaker mechanism of the heart, as Prosser (1940) found in *Venus*. This statement is con-

firmed by the fact that the *Ariolimax* heart, brought to diastolic stoppage by acetylcholine, reacts to direct local stimulation with a strong contraction; the contractile mechanism is therefore intact, but the pacemaker is impaired. Acetylcholine must also have an effect on the mechanism of contraction, for it produces a decrease in amplitude of beat; this may indicate a slight impairment of this mechanism. That this mechanism is not completely impaired is shown by the effect of electrical stimulation as cited above.

The effect of eserine in lowering the acetylcholine threshold indicates the existence of cholinesterase in the heart tissue.

Nicotine seems also to act upon the pacemaker mechanism, since at a low concentration it slows the heart; it also may affect the mechanism of contraction, since the amplitude is increased. At higher concentrations, it stops the beat with the ventricle in diastole; that this stoppage is not due to an impairment of the mechanism of contraction is shown by the fact that the heart reacts to direct electrical stimulation with a strong contraction. The phenomenon of grouped beats leading to the normal rhythm during recovery from nicotine treatment may be interpreted as the result of a gradual recovery of the pacemaker mechanism.

The experiments with acetylcholine on the nicotinized heart (Figs. *2D*, *3A*, *3B*) present evidence that the two drugs have much the same effect on the pacemaker, though they apparently do not on the mechanism of contraction; concentrations of acetylcholine not high enough to affect the normal heart produce characteristic inhibitory effects on the nicotinized heart. The grouped beats observed in the nicotinized heart during treatment with acetylcholine 1 : 10 million (Fig. *2D*) may therefore possibly be explained by assuming that the pacemaker mechanism, already affected by nicotine, was placed on the verge of cessation of activity (which would result in diastolic stoppage of the heart), a condition approximating that which existed during recovery from diastolic stoppage by nicotine 1 : 4000 (Fig. *2C*).

One of the most fundamental observable differences in the response to drugs of molluscan and vertebrate hearts is the inability of atropine to block acetylcholine action in the former; this phenomenon was verified in *Ariolimax*.

Can it be said that the action of acetylcholine on the slug heart is "nicotine-like"? Atropine certainly fails to abolish its effects. The tests would seem to show that at least as far as the pacemaker is concerned, the effects of acetylcholine and nicotine are similar and additive. But the effects of the two drugs on the mechanism of contraction are certainly not the same.

ASTACUS TROWBRIDGEI

Brief preliminary tests were made on the heart of this animal with acetylcholine and other drugs. An attempt was made to corroborate the results obtained by MacLean and Beznak (1933), using the heart of *Astacus fluviatilis*, and to compare results with those obtained by Welsh testing the hearts of *Panulirus* and *Carcinus* (1939*a*, 1939*b*).

After the crayfish was pithed, a section of the carapace above the heart was removed and tissue surrounding the heart carefully trimmed away. Coagulation of the blood was prevented by immediate irrigation with physiological solution. A writing arm was then attached directly to the heart by means of a glass hook. Drug solutions were introduced directly into the cavity around the heart. By this technique a constant internal pressure was not maintained in the heart; however, in a number of tests results of considerable interest for comparison with those of other investigators were obtained. The heart of *Astacus* is delicate and sensitive to manipulation, but it is hoped that better records may be obtained by perfusion according to the technique of Cole et al. (1939) or by isolating the heart according to the technique of Welsh.

Drug solutions were made up from stock in Van Harreveld's solution: 12 grams NaCl, 1.5 grams $CaCl_2$, 14 grams KCl, .25 gram $MgCl_2$, (.29 gram $NaHCO_3$ per liter), plus distilled water to a liter.

All experiments conducted and solutions used were at room temperature (15°–20° C.).

Acetylcholine

There was found to be considerable individual variation in sensitivity to the mediator in these autoperfused hearts. However, acetylcholine 1:10,000 produced in a number of individuals a powerful increase in frequency and amplitude. In no tests with this concentration was there produced "a tonus increase . . . accompanied by a reduction in amplitude of beat due to incomplete recovery between strokes" or stoppage of the heart in systole (Welsh, 1939*a*).

Apparently MacLean and Beznak (1933) did not test concentrations of acetylcholine lower than 1:1,000,000. It was found that the heart of *Astacus trowbridgei* was insensitive to concentrations lower than 1:10,000,000,000 but at this concentration, which may be taken as the threshold, there was in a number of tests a noticeable increase in frequency. Welsh found that the isolated heart of *Carcinus* is insensitive to concentrations lower than 1:100,000,000 and that of *Panulirus* to concentrations lower than 1:10,000,000,000, which figure has been taken as the approximate threshold for the *Astacus* heart. A comparison of

these figures with the figures given for Mollusca in Table I will show that Prosser's (1940) statement based on his study of *Venus* that "the threshold for the accelerating action on arthropod hearts is higher than that for inhibition in the molluscan hearts" is not generally true.

Eserine

Welsh's tests with eserine on the isolated heart of *Panulirus* showed that with eserine 1 : 100,000 "if the heart had been beating in a somewhat irregular manner (e.g. exhibiting a series of grouped beats), a regular beat would be completely restored." This was found to be true also for the *Astacus* heart. If the heart had been beating regularly, administration of eserine often slightly increased the amplitude.

In a test during which eserine 1 : 100,000 was administered continuously the amplitude at first slightly increased, but later as the frequency also increased the amplitude of beat became less. With the inactivation of cholinesterase present in the heart one might expect somewhat the same results as produced by the administration of acetylcholine because of the sensitization of the heart to small amounts of the mediator that may be present.

Atropine

As has been shown for other arthropods, the stimulatory action of acetylcholine is abolished by atropine. In the crayfish tests showed that both amplitude and frequency effects of 1 : 10,000 acetylcholine are abolished by previous treatment with atropine 1 : 10,000.

Nicotine

The effect of nicotine on the *Astacus* heart seems similar to that on the heart of *Ariolimax*. In tests nicotine 1 : 10,000 produced a marked increase in amplitude and at the same time, if the heart was beating in Ringer with a swift regular beat, produced a slight decrease in frequency. At the higher concentration of 1 : 5000 which must approach toxicity, there occurred marked inhibition of both frequency and amplitude.

Adrenaline

Welsh showed that in *Panulirus* adrenaline in high concentrations had a powerful stimulatory effect of a different type from that of acetylcholine. In *Astacus* adrenaline 1 : 100,000 produces a violent acceleration and increase in amplitude and as in *Panulirus*, although the frequency rapidly returns to normal, the amplitude effect remained for some time after replacement by Ringer.

Although MacLean and Beznak (1933) say that in *Astacus fluviatilis* they were able to produce only a slight increase in frequency with adrenaline 1:1000, in *Astacus trowbridgei* concentrations of 1:1,000,000 produce a sharp increase in frequency without changing the amplitude.

Conclusions

Can it be said that the action of acetylcholine on the crayfish heart is "muscarine-like"? Atropine abolishes its effects. As has been stated, the effects of nicotine on this heart do not resemble the effects of acetylcholine on it, but they are remarkably similar to the effects produced by nicotine on the slug heart.

It is too early to conclude whether or not these two actions of acetylcholine, the "nicotine-like" and the "muscarine-like" well known in the vertebrates, really are found among the invertebrates. It is hoped that future experiments may cast light on this problem.

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